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STRATEGIES FOR MUTATION DETECTION IN SEX-CHROMOSOME RELATED DISORDERS

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**A Thesis Submitted to the Faculty of Medicine, University of
Glasgow, for the Degree of Doctor of Philosophy**

**Duncan Guthrie Institute of Medical Genetics,
Yorkhill Hospitals NHS Trust
Glasgow**

August 1999



Declaration

I certify that this thesis does not contain material previously published or written by any other person, except where referred to in the text. The results in this thesis have not been submitted for any other degree or diploma.

Fawziah M. Mohammed

Acknowledgements

During this work, I have benefited greatly from the generous advice and help of many experts and friends.

I would like to thank Professor J.M. Connor for the opportunity to pursue this course at the Duncan Guthrie Institute of Medical Genetics, University of Glasgow, and for his supervision and help through this work. I am equally indebted to my supervisors Dr. W.G. Lanyon and Dr. E. Boyd for their guidance and encouragement during the course of this study. I would also like to thank Dr. C. Chu for her help, valuable suggestions, and for providing samples. I am also thankful to Dr. A. Chandley for her generous gifts of probe MK5 and MK29. I would like to thank Dr. Ahmed Al-Ansari at male infertility clinic-Kuwait for his help in patient assessment and sampling.

I am very thankful and grateful to Eqbal El-Rahmani, Mahbuba Hagig, Alaa Elshafey, Guity Ghaffari and Riza Davoodi for being devoted friends and allies and for their help, wisdom and criticism. I am especially grateful to all my friends Manal Bohaimed, Lubnah and Iman Al-Behnasawi, Sawsan Abulhasan, and Iman Abohamra who offered encouragement and moral support during my stay in Glasgow.

My gratitude is also extended to Dr. Sadika Ali Al-Awadi Director of Kuwait Medical Genetics Center for support, guidance, and being very patience.

I am equally thankful to Dr. Laila Bastaki Dr. Sayed Guoda, Dr. Rizk Al-Naggar and to all staff of Kuwait Medical Genetics Center for their moral support.

Special thanks and appreciation to Dr. Kamal Naguib, Dr. Krishna Murthy, Dr. Dr. Shawki Tayel, Khaled Al-Nassafi, and Dr. Maha Mustafa Abo-Hendi at Kuwait Medical Genetics Center for their guidance and patience during the preparation of this thesis.

I am grateful to the post graduate research (PGR) laboratory group especially Morteza Bonyadi, Mojtaba Mohaddes, Hassan Acar, Munis Dundar, Huseyin Yuce and Eunice Stefanous.

Many thanks to all staff at Duncan Guthrie Institute of Medical Genetics for being kind and for their help and valuable advice particularly Betty O'Hare, Ann Theriault, Norma Morrison, Avril Morris, Sandy Cooke and Jim Colgan.

And of course, I shall always be grateful and obliged to my parents for their love and support particularly during my long stay away from home. I am also very thankful and indebted to all my brothers and sisters for their faith, love, and encouragement and who have been there whenever I needed them.

Finally I would like to thank all the patients who participated in the research. Without them the work would not have been possible.

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LIST OF ABBREVIATIONS

| | |
|--------------------|---|
| AIRS | artificial introduction of restriction site |
| AIS | androgen insensitivity syndrome |
| AR | androgen receptor |
| ARMS | amplification refractory mutation system |
| ASO | allele specific oligonucleotide |
| AZF | azoospermia factor |
| Biotin-11dUTP | biotin deoxyuracil 5`-triphosphate |
| bp | base pair |
| Brdu | bromodeoxy uridine |
| BRL | Bethesda Research Laboratory |
| CAP | chromosome arm paint |
| cDNA | complementary DNA |
| CFTR | cystic fibrosis transmembrane regulatory gene |
| CISS | chromosomal in situ suppression |
| cm | centimetre |
| CCM | chemical cleavage of mismatches |
| cpm | count per minute |
| DAPI | 4,6-diamino-2-phenl-iodole |
| dATP | deoxyadenosine 5`-triphosphate |
| DAZ | deleted in azoospermia |
| dCTP | 2`deoxycytidine 5`-triphosphate |
| ddH ₂ O | double distilled water |
| ddNTP | dideoxyribonucleoside triphosphate |
| DGGE | denaturing gradient gel electrophoresis |
| dGTP | deoxyguanosine 5`-triphosphate |
| dH ₂ O | distilled water |
| DMD | duchenne muscular dystrophy |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside triphosphate |
| DSS | dosage sensitive syndrome |

| | |
|-----------|---|
| DTT | dithiothreitol |
| dTTP | deoxythymidine 5'-triphosphate |
| EDTA | ethylenediamine-tetra-acetic acid |
| EMC | enzyme mismatch cleavage |
| FISH | fluorescent in situ hybridisation |
| FITC | fluorescein isothiocyanate |
| FRAX | fragile X |
| FSH | follicle stimulating hormone |
| g | gravitational (centrifugal) force |
| G-banding | Giemsa banding |
| GBY | gonadoblastoma gene on Y chromosome |
| gm | gram |
| HA | heteroduplex analysis |
| HMG | high mobility group |
| ISCN | International System for Chromosomal Nomenclature |
| ISH | in situ hybridisation |
| IU | international units |
| KAL | Kallman |
| kb | kilobase |
| kd | kilo dalton |
| l/L | litre |
| LH | luteinising hormone |
| M | molar |
| Mb | megabase |
| mg | milligram |
| MIS | Mullerian inhibiting substance |
| ml | millilitre |
| mM | millimolar |
| MRC | Medical Research Council |
| mRNA | messenger ribonucleic acid |
| nm | nanometer |
| NOR | nucleolar organizer region |
| °C | degree centigrade |
| OD | optical density |

| | |
|-----------------|---|
| ORF | open reading frame |
| p | short arm of any chromosome |
| ³² P | radioactive phosphorus |
| PAGE | polyacrylamide gel electrophoresis |
| PAR | pseudo autosomal region |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PHA | phytohaemagglutinin |
| pmol | pico mole |
| q | long arm of any chromosome |
| Q-banding | quinicrine banding |
| RBM | RNA-binding motif |
| RFLP | restriction fragment length polymorphisms |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RPS4 | ribosomal protein S4 |
| RT-PCR | reverse transcription polymerase chain reaction |
| SDS | sodium dodecyl sulphate |
| SMA | spinal muscular atrophy |
| SRY | sex determining region on the Y |
| SSC | saline sodium citrate |
| SSCP | single strand conformation polymorphism |
| STS | sequence tagged site |
| TAE | tris-acetate ethylenediamine tetra-acetic acid |
| Taq | Thermus aquaticus |
| TBE | tris-borate ethylenediamine tetra-acetic acid |
| TDF | testis determining factor |
| TE | tris-ethylenediamine tetra-acetic acid |
| TEMED | N,N,N,N-tetramethylethylenediamine |
| TGGE | temperature gradient gel electrophoresis |
| T _m | melting temperature |
| TS | Turners syndrome |
| u | unit |
| UV | ultraviolet |

| | |
|------|---|
| WCP | whole chromosome paint |
| WT | Wilm's tumor |
| XIST | X-inactivation specific transcript |
| YRRM | Y chromosome RNA recognition motif |
| μg | microgram |
| μ l | microlitre |
| μM | micromolar |
| PAR | pseudoautosomal region |
| ZFY | zing finger on Y chromosome |
| TSPY | testis specific protein on Y chromosome |

SUMMARY

The aims of the present study were to optimize and apply methods that could detect major or minor DNA sequence alterations in patients with sex chromosome aberrations, with sex determination/differentiation abnormalities and in patients with idiopathic male infertility. These strategies involved the application of cytogenetic, molecular cytogenetic (FISH) and molecular genetic investigations. For each of the groups studied a different mutation strategy (or strategies) was chosen. A total of 93 cases have been included.

Unicolour, dual colour and multicolour FISH analyses were successfully optimised using different probe concentration, competitor DNA concentrations, hybridisation temperature, and detection methods. The optimal final concentration of the DNA mixture (probe and competitor DNA)/2 cm² hybridisation was found to be between 1-300 ng of the probe and 5-12 µg of the competitor DNA. The optimal hybridisation temperature was between 37-42 °C for 14-16 hours incubation time. A total of 21 probes were used, 15 were specific for chromosome X and six were specific of the Y-chromosome. All probes used in this study were optimised to provide strong and well-defined signals on metaphase and interphase spreads.

Among patients with sex chromosome aberrations, the optimised biotin- and/or digoxigenin-labelled X- and Y-chromosome specific DNA probes were applied to characterise the abnormalities in the sex chromosomes. Three general types of probes were used for FISH-based cytogenetic analysis; whole chromosome probes, chromosome specific repeats, and probes targeted to specific loci. They were used separately or in combination. Late replication (X-inactivation) study using BrdU was optimised and applied for structurally abnormal X chromosomes particularly ring X and X-autosome translocation.

For patients with sex determination/differentiation abnormalities and with male infertility, conventional cytogenetics methods were applied. Optimised FISH and different molecular techniques including multiplex PCR, SSCP, heteroduplex analysis, CCM analysis, Southern blot and DNA sequencing were applied for the detection and full characterisation of mutation(s) in the androgen receptor (*AR*), *SRY* and *RBMI* genes.

Out of the 93 patients, 24 patients had sex chromosome aberrations diagnosed by conventional cytogenetics. Most of them were ascertained because they had Turner syndrome/variant phenotypes. Reassessment of the chromosomal constitution of those patients using fluorescent in situ hybridization technique revealed that in 20 patients (83.3%) the FISH results were consistent with the cytogenetic findings. However, FISH analysis has confirmed the presence of mosaicism and/or structural abnormalities, delineated the breakpoints, and identified the origin of the marker chromosome among them. In addition, FISH investigation has detected cryptic rearrangements in four patients (16.7%). One patient diagnosed i(Xp) was found by FISH analysis to be t(Xp;Xq). A second patient with 46,XXp+ was precisely diagnosed as t(Xp;16q). A third patient with marker chromosome was delineated as t(X;Y) and the fourth patient diagnosed by cytogenetics as mar(?der Y) has proved by FISH to be i(Yq), in addition to the detection of a third cell line in the form of r(Y). Late replication study using BrdU in cases of ring (X) showed active normal X while the ring (X) was either early replicating or late replicating within the same case. No genotype-phenotype correlation could be made in these cases in relation to the ring size or its replication pattern. In case of t(Xp;16q), the derivative X was confirmed to be late replicating in all cells examined. However, the translocated segment of 16q demonstrated a differential replication pattern, which could be correlated with the patient phenotype.

In the remaining patients, 13 had sex determination / differentiation abnormalities and 56 had infertility problems.

The molecular screening methods used in this study included chemical cleavage mismatch analysis (CCM), single strand conformational analysis (SSCP), multiplex PCR, Southern blotting and DNA sequencing. The strategy of using DNA as template for PCR and use of SSCP, heteroduplex and DNA sequencing analysis as the main screening methods has proved to be a useful screening strategy for detection of mutations within *AR*, *SRY*, and *RBM1* gene. Molecular genetic analysis for diagnostic purposes employing SSCP has proven to be a very useful approach for a variety of diseases.

Among the 5 cases of androgen insensitivity syndrome (46,XY female), a point mutation in the steroid binding domain (exon 8) of the *AR* gene was detected by PCR-SSCP analysis in one patient with the complete form of the disease. The

substitution resulted from a point mutation at nucleotide 4109 G→T causing replacement of Val 889 by Leu.

Molecular analysis of the *SRY* ORF, promoter and the poly-A tail regions in 4 patients with either complete or partial 46,XY gonadal dysgenesis has revealed no mutations in this region suggesting that these patients have a normal *SRY* gene. However, the failure to detect mutations in these unrelated cases does not exclude the existence of mutations.

In the present study, cytogenetic analysis, FISH and molecular amplification have been used to detect the presence or absence of Y sequences and to characterise the transfer of Yp fragments to Xp22.3 in 4 XX males. FISH techniques using GMGY10 and hoSRY probes specific for the Yp11.2 region were applied on the metaphase spreads and revealed the presence of the Yp11.2 sequences on the short arm of the X chromosome in three patients while it was absent in the fourth one. DNA analysis of these 4 patients using *SRY*, *ZFY*, *PAR* and *Y1/Y2* sequence amplification confirmed the existence of the *SRY*, *AZF*, and *PAR* sequences in three patients and its absence in the fourth patient.

Fifty-six cases with male infertility were first analysed by routine cytogenetics. Abnormal karyotypes were found in 5 cases (8.9%). Four cases (7.1%) had 47,XXY and one case had 47,XYY. Out of the 50 men with idiopathic infertility only one azoospermic male (2%) was found to have a Y chromosome microdeletion by multiplex PCR analysis. Six PCR products were absent in 4 out of 5 multiplex reactions. These deletions involved sY153 (subinterval C), sY147 and Y6HP52pr (subinterval D), sY 157 and sY 149 (subinterval E), and Fr15-11pr (not determined) and the breakpoints in Yq11 was estimated to be in subinterval MX-MXII of the Ma map between interval 6C and 6F, according to the Vollrath deletion map. PCR study using *DAZ* specific primers showed that all infertile patient and controls retained the *DAZ* gene, yet it was deleted in the patient with microdeletions. The father of the patient also retained *DAZ* gene. Therefore, the patient in this study has a *de novo* microdeletion in Yq11 region, which spanned the *DAZ* gene region. Moreover, FISH analysis and Southern blot hybridisation to DNA from 50 infertile men and 50 controls using cDNA encoding *RBM1* and *RBM2* respectively revealed the multiple copies of the gene without any deletions or structural rearrangements. Accordingly, the possibility of the deletion or rearrangement of the *RBM* gene family was ruled out as a cause of the infertility in these cases. Finally, the DNA of 50 idiopathic

patients with normal karyotypes was amplified with intronic primers specific for exons 1-12 of the *RBM1* genes. Single strand conformation polymorphism (SSCP) analysis was optimised to screen exons 1-11 and heteroduplex analysis (HA) was optimised to screen exon 12 of the gene in all cohorts of the studied patients. No small deletions or structural rearrangements were detected in all cohorts, including the patient who showed microdeletion in Yq11 upon multiplex PCR experiments. The possible reasons for these findings are, (1) the responsible gene (*RBM*) may be unimportant in the process of spermatogenesis. (2) Point mutations may not be present, or not detectable, or in regions of the gene not yet analysed in these patients. (3) *RBM* is a multicopy family of genes and hence mosaicism is not easily shown. Finally, in this study no *de novo* point mutations were found which might have indicated that the *RBM* genes are involved in the failure of spermatogenesis in all investigated patients.

CHAPTER 1

INTRODUCTION

(1) INTRODUCTION

The genetic information is carried by discrete entities called “chromosomes”, a characteristic number of which exist for each species. In man there are 46 chromosomes: 22 pairs (number 1-22) of autosomes and one pair of sex chromosomes (XX in females, XY in males). The fundamental dogma of molecular biology is that deoxyribonucleic acid (DNA) codes for ribonucleic acid (RNA) which in turn produces proteins. Thus the genetic information in the DNA specifying a particular function is converted into an RNA copy which is then translated into protein. The action of the protein then produces the phenotype. Phenotypic variation, both normal and abnormal differences, can be considered in terms of several aetiologies: (1) Cytogenetic disorders, which result from changes in the chromosome number (deviation from the normal number) or structure (deviation in chromosomal morphology). The former arises by chromosome malsegregation and the latter arise by either centromeric misdivision or chromosome breakage. (2) Mendelian disorders, which result from a mutation at a single genetic locus, causing an abnormal allele. (3) Polygenic (multifactorial) disorders which could result from (a) the cumulative effect of several different genes, (b) multiple alleles at a single locus, or (c) a combination of genetic and environmental factors and (4) teratogenic agents (Weatherall, 1991; Connor and Ferguson-Smith, 1991).

1.1 Molecular pathology of single gene disorder

Any trait caused by a single gene mutation is said to be Mendelian, or to have single gene inheritance. Such traits can be divided into autosomal dominant, autosomal recessive, sex linked and mitochondrial (Thompson et al, 1991). A gene can be disrupted in many ways. Coding regions may be altered, promoter and other regulatory sequences may be disturbed, or processing signals such as splice sites may be modified. Alterations may vary from deletions or rearrangements (insertion, inversion, and duplication) that can remove an entire gene to individual nucleotide changes (single base-pair substitution). These various types of

mutations have been detected and characterised in many human genes, thus enabling the study of the underlying mutational mechanisms.

Nucleotide changes may or may not cause altered amino acid synthesis. Point mutations have been classified into silent, missense, nonsense and sense. In silent mutation, base substitution causes no change in the amino acid therefore it has no consequence at the phenotypic level. The protein product of the mutant will function just as well as that of the wild type. In missense mutation, a codon change results in coding of different amino acid. The effect of this mutation on protein function depends on its location in the protein molecules. In nonsense mutation, a stop codon (UAA, UAG, or UGA) is created, causing premature termination of protein. In sense mutation, a termination codon is changed to one that codes to amino acids and results in the translation of protein beyond the normal termination signal until another termination codon is reached producing longer than normal protein (Weatherall, 1991).

Nucleotide substitutions are the most common type of mutations in coding DNA sequence. Nucleotide changes that involve the substitutions of one purine for another ($A \leftrightarrow G$) or one pyrimidine for the other ($T \leftrightarrow C$), called transition, are more frequent than transversion (substitution of pyrimidine by purine or vice versa). The excess of transition can be explained with finding that major form of DNA modification in the human genome involves methylation of cytosine residues to form 5'-methylcytosine, predominantly in CpG dinucleotides (Cooper and Krawczak, 1993). Spontaneous deamination of 5'-methylcytosine to thymine in CpG dinucleotides gives rise to $C \rightarrow T$ or $G \rightarrow A$ transition. Since 35% of single base pair mutation occurred within CpG dinucleotides with transition substitution occurring with a frequency of over 90% (Cooper and Krawczak, 1991), the CpG dinucleotides appears to be a hotspots for mutation and contributes significantly to the molecular pathology of many disorders.

One important aspect of RNA processing is the precise splicing out of introns to produce a mature mRNA ready for translation. Nearly all eukaryotic genes have a dinucleotide GT at the 5' starting point of the intron (the donor site) and a dinucleotide AG at the 3' (the acceptor site). There are a wide variety of mutations in introns that affect normal RNA splicing (Krawczak et al, 1992). Naturally occurring mutations that affect mRNA splicing can be assumed to fall into three main categories: (1) mutations within a 5' or 3' splice site. Such lesions

usually reduce the amount of mature mRNA generated and/or activate so-called cryptic (i.e. alternative) splice sites resulting in the production of mRNA which either lacks a portion of the coding sequence (exon skipping) or which instead contain additional sequences of intronic origin (cryptic splice site situation). According to the definition model proposed by Robberson et al (1990), exons are recognised and defined as distinct units by the initial binding of protein factors to the 3' end of the intron followed by a search for a down stream 5' splice site. Thus an alternative consequence of splice site mutation could be for an exon to be no longer recognised as such, and as a result to be excluded from the mature mRNA transcript- a process termed exon skipping. (2) Mutations within an intron. This kind of mutation in the introns includes those that cause the activation of cryptic splice sites, by making a sequence to be more similar to an authentic consensus site or by creating new splice sites leading to the production of aberrant mRNA species. (3) Mutations within a branch-point sequence (Krawczak et al, 1992).

Alterations of gene structure by deletion, insertion or duplications have been described in various inherited disorders and the frequency of such mutations differs greatly among different genetic diseases. Deletions may be the underlying cause of single gene Mendelian disorders. Deletions of multiple exons may remove a portion of the gene products, if newly juxtaposed exons are not in frame, a truncated product may result (Monaco AP, 1989). Gross gene deletions may arise either by homologous unequal recombination mediated by related gene sequences or repetitive sequences, or by non-homologous sequences involving DNA with minimal sequence homology (Krawczak and Cooper, 1991). Smaller deletions may remove specific amino acids from a protein if the deletion involves multiples of three bases. Other deletions result in frame shift that usually truncates the protein product. Direct repeats palindromes and symmetric elements could be potential mechanisms involved in gene deletions.

Duplications involving part or all of a gene can cause genetic disorders because the inserted material disrupts the reading frame of a gene, leading to premature termination of translation. Gene duplications may also occur due to homologous or non-homologous recombination. For instance, homologous recombination may arise between repetitive sequences such as *Alu* elements however, non-homologous recombination may cause in duplication by random chromatid breakage and rejoining events.

Insertion of < 10 base pairs (bp) sequences into human genome coding region were also investigated by Cooper and Krawczak (1991). They stated that this event is not random and appears to be highly dependent on the local DNA sequence context. The majority of insertion can be explained either by (1) mispairing, or by (2) inverted repeats or by (3) symmetric elements (e.g. CTGAAGTC, GGACAGG) during DNA replication.

1.2 Sex determination and sex differentiation

The existence of males and females is due to two processes: sex determination and sex differentiation. Sex determination is the process of choosing the male or female differentiation pathway (Goodfellow and Lovell-Badge, 1993). Sex determination and differentiation are sequential and complex processes starting with the establishment of genetic sex at conception, followed by gonadal determination in early embryogenesis and ends in puberty with complete sexual maturation and the ability to reproduce (Moore and Grumbach, 1992). The critical factor in this process is the differentiation of the bipotential genital ridge into either a testis or an ovary. Sex differentiation is governed by a pathway involving X-linked and autosomal genes and the pathway is initiated by a dominant inducer Y specific master gene called TDF (Testis-determining factor). Male sex determination is dependent on the presence of a Y-chromosome and TDF (Page et al, 1987). Male gonadal sex or testis formation is subsequently thought to be determined by this gene and by other secondary pathways. The male gonad in turn normally produces hormones that regulate differentiation of the internal and external genital structures, thus determining phenotypic sex. In the absence of the Y chromosome (and TDF), the foetal gonads follow the ovarian pathway, with ensuing female development (Ford et al, 1959). When an abnormality develops in any of the above three processes complete or incomplete sex reversal individual results due to the discrepancy in genetic, gonadal and phenotypic sex (Gustafson et al, 1994).

1.2.1 Normal sex differentiation

The early embryo has no sexual characteristics except for the chromosomal complement of its cells. In human embryos, the gonadal development begins

during the 4th and 5th weeks of gestation. The complete differentiation of normal gonads depends upon the arrival of sufficient numbers of viable primordial germ cells to the genital ridges of the dorsal mesentery. The germ cells arise extragonadally and therefore must migrate into the site of gonadal differentiation. Seeding of the ridges by primordial germ cells is an independent event not requiring Y chromosome and TDF (Moore and Grumbach, 1992). If they fail to arrive, gonads do not develop (gonadal agenesis) and only a fibrous streak will exist. At 6 weeks of foetal life, the indifferent gonad is bipotential, capable of forming either testis or ovary.

Before gonadal differentiation, Wolffian ducts have already developed (by 4 weeks). Mullerian ducts appear at about 7 weeks. In an XY embryo (normal male) the testis-determining switch commits the primitive gonads to testicular development, resulting first in Sertoli and later in Leydig cell differentiation. Sertoli cells envelope the primordial germ cells and form primitive cords (precursors of the seminiferous tubules), and inhibit germ cell meiosis. Their differentiation is predominantly cell autonomous and requires a Y-chromosome (Moore and Grumbach, 1992). The developing testis secretes two hormones that are important for the subsequent male differentiation. Mullerian-inhibiting substance (MIS) produced by Sertoli cells prevents formation of the uterus, fallopian tubes and upper part of the vagina (Mullerian structures) (Josso and Piccard, 1986). Leydig cell testosterone secretion is responsible for persistence of the Wolffian ducts and their differentiation into epididymis, vas deferens, seminal vesicles and ejaculatory ducts, and promotes the masculinisation of the external genitalia. The latter step requires conversion of testosterone to dihydrotestosterone (DHT) (Siiteri and Wilson, 1974) and the subsequent interaction of DHT with intracellular receptors (androgen receptors) (Migeon et al, 1981). DHT is responsible for fusion of labia, growth of the phallus and formation of scrotum. Wolffian development and masculinisation of the external genitalia begin at about 10 weeks and are completed by 16-17 weeks (Marcantonio et al, 1994).

In the XX (normal female) individual, without the active influence of a Y chromosome, the bipotential gonad is committed to ovarian development. The gonad is silent until about 12 weeks when oocytes begin to differentiate and primordial follicles are present by 20 weeks. In the absence of Leydig cell testosterone, the Wolffian ducts virtually disappear completely. The Mullerian

system, in the absence of Sertoli cell MIS secretion, differentiates into upper vagina, uterus and fallopian tubes.

1.2.2 Role of chromosomes in sex determination

It has been known for decades that sex determination in mammals is accomplished by a chromosomal mechanism. Females have two X chromosomes, and males have a single X and a small mainly heterochromatic Y chromosome. Analysis of individuals with unusual sex chromosome combination has shown that the presence of the Y chromosome is critical for male sex determination and absence of the Y chromosome results in female development. Ford et al (1959) showed that individuals with Turner syndrome had a single X chromosome and were females while patients with Klinefelter syndrome were shown to have two X chromosomes and a Y, and were males (Jacobs and Strung, 1959). These results implied that the Y chromosome carries a gene required for testis development. The concept that genes located on the Y chromosome play an essential role in human sex determination is stressed by the existence of various sex chromosomal aberrations. These conditions include the 46,XX male syndrome, true hermaphrodites, 46,XY gonadal dysgenesis, and various Y chromosomal aberrations that result in female development. From the analysis of Y chromosome rearrangements and their associated phenotypes, the testis determination factor (TDF) has been assigned to the short arm of the Y chromosome (Davis RM, 1981), and more precisely mapped to the most distal specific segment adjacent to the pseudoautosomal boundary (Affara et al, 1986; 1987, Vergnaud et al, 1986).

There are two features that make sex chromosomes unique in mammalian genome. Most obviously, they determine sex. Also, they are morphologically and genetically different from each other, a difference that has accompanied the appearance of a sex determining role on the Y chromosome and a mechanism of dosage compensation for X-linked genes in females, mediated by random X inactivation (Graves JAM, 1995).

The Y chromosome is composed of two parts with different proportion: the pseudo autosomal region (PAR), shared with the X chromosome, and the Y-specific region. At male meiosis the Y chromosome pairs with the X chromosome and the pairing occurs within the PAR region shared between the short arms of the

X and Y chromosomes (Burgoyne PS, 1986; Chandley et al, 1984). An obligate recombination event within the PAR is necessary to ensure correct meiotic segregation (Burgoyne PS, 1982). The Y-specific regions include the sex determining factor (TDF) region containing the *SRY* gene, located proximal to the PAR, the putative transcription factor ZFY (Zinc finger on Y), the gene for H-Y antigen (serological histocompatibility Y), genes necessary for spermatogenesis (Buhler EM, 1980; Goodfellow et al, 1985), anti-Turner gene (RPS4; ribosomal protein S4) (Fisher et al, 1990) and genes implicated in the development of gonadoblastoma (GBY) (Page D, 1987; Verp and Simpson, 1987; Barbosa et al., 1995). On the distal portion of the long arm of the Y chromosome is the heterochromatic region, which is apparently not transcribed and varies in length (Fig1.1).

The X chromosome is currently the best characterised among all human chromosomes (McKusick VA, 1994). With the exception of genes located in the PAR (Ellis and Goodfellow, 1989), and a few other genes which have active copies on both the X and Y chromosomes, most genes on the X chromosome display a haploid status in males (Ballabio and Andria, 1992). The X chromosome is significantly larger than the Y chromosome but organised in a similar fashion, having a pseudo autosomal region matching that of the Y and an X-specific region (Fig 1.1). The X-specific region contains many genes including some genes crucial for male and female sexual differentiation and other genes that are essential for life (45,Y is lethal) (Moore and Grumbach, 1992).

Because XX females have twice as many X-linked genes as XY males, a dosage compensation mechanism is achieved by X chromosome inactivation in order to avoid the aneuploidy effect that would stem from the presence of more than one X chromosome (Lyon M, 1961; Riggs and Pfeifer, 1992). The inactivation of one X chromosome is thought to be an essential part of female sex differentiation. X inactivation is associated with a switch in the timing of chromosomal replication, the inactive X chromosome being the last to initiate DNA synthesis (Lyon M, 1992). This late replicating chromosome is relatively condensed during the interphase and is visible cytologically as a Barr or sex chromatin body at the nuclear periphery (Migeon B, 1994).

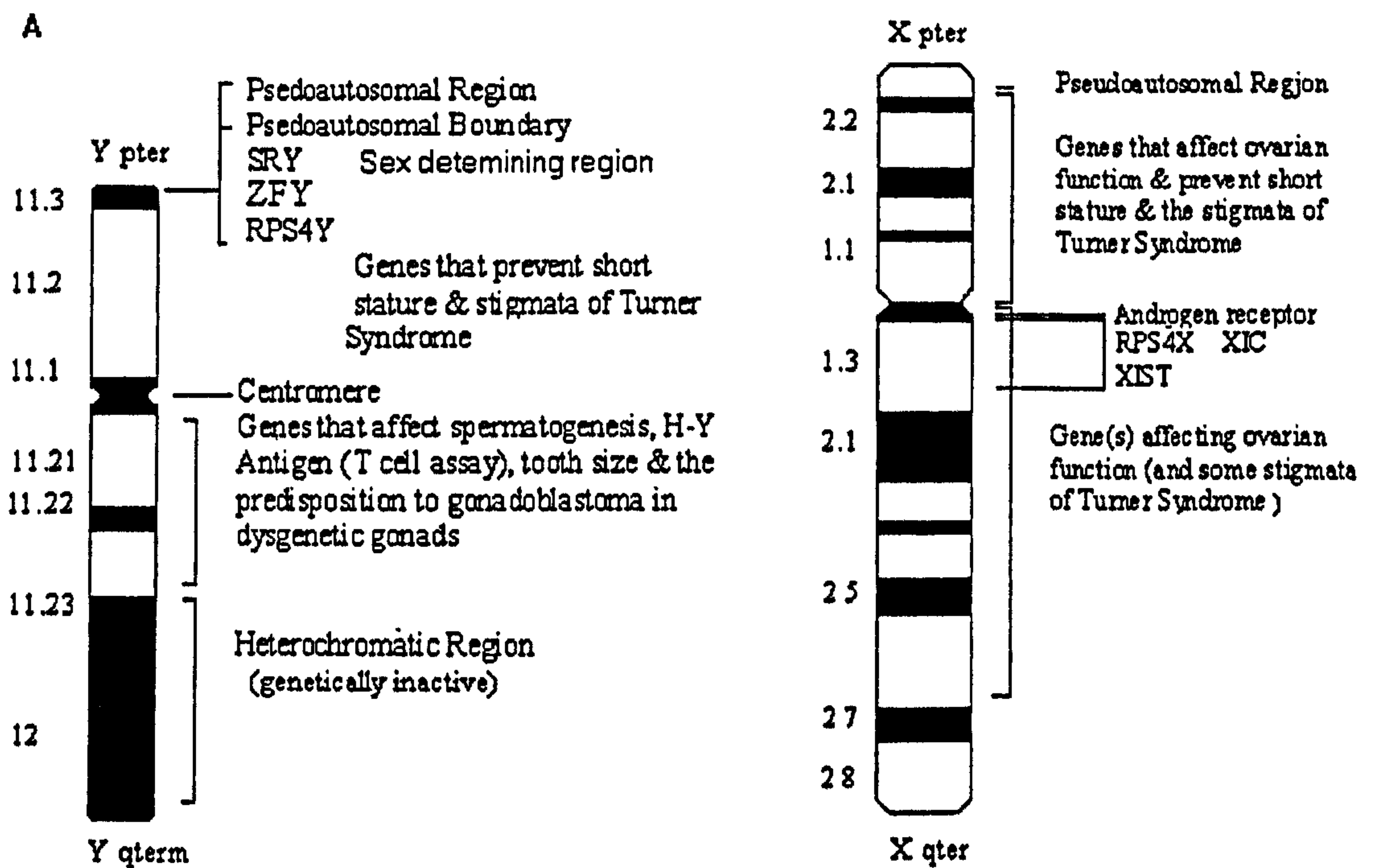


Figure 1.1: Diagrammatic representation of the G-banded Y and X chromosomes. Functional regions and loci involved in sex determination and differentiation are shown. (A) The Y chromosome: SRX= sex determining region; RPS4Y= ribosomal protein S4; ZFY= zinc finger protein. (B) The X chromosome: RPS4X= ribosomal protein S4; XIST= X-inactive specific transcript; XIC,= X-inactivation center.

The X inactivation begins early in female embryological development and it occurs randomly with respect to parental origin of the chromosome. Once silenced the inactive X chromosome usually remains silent in all progeny cells (clonal inheritance). Although inactivation seems to affect the X chromosome as a whole (inactivating many loci), some loci on the inactivated human X chromosome are actually transcribed. These transcribed genes include not only those in the pseudo autosomal region (Xp22.3), but also some of the proximal short arm and the proximal long arm of the X chromosome such as zinc finger X (ZFX at Xp22.1) and ribosomal protein S4 (RPS4X at Xq13) (Fisher et al, 1990; Schneider et al, 1989; Disteche et al, 1995). Furthermore, in female germ cells (oogonia) only one X chromosome is active but the inactive X is reactivated at about the time of entry into meiosis (Migeon and Jelalian, 1977; Monk and McLaren, 1981). Therefore both X chromosomes are thought to be active during oogenesis itself. On the other hand, in spermatogenesis the single X chromosome is inactive (Monk and McLaren, 1981).

The precise nature of the X inactivation, its control mechanism and sequence of imposition is still unclear. However, genetic evidence strongly suggests the existence of a specific locus required in *Cis* for X inactivation located within a segment of the X chromosome referred as the X-inactivation centre (XIC) (Borsani et al, 1991). This XIC region has been localised to the long arm of the X chromosome at Xq13 (Therman et al, 1990; Brown et al, 1991). Recently, a gene has been isolated and mapped to Xq13 and proposed to be a candidate for the inactivation centre. It is referred to as *XIST* (X-inactivation specific transcript) in human (Brown et al 1991) and *Xist* in mouse (Borsani et al, 1991; Brockdorff et al, 1992) and is expressed from the inactive but not the active X chromosome. The complete cDNA sequences of the human (Brown et al, 1992) and mouse *XIST* (Brockdorff et al, 1992) have been determined and no substantial open reading frame has been found, and the RNA is confined to the nucleus. It has been suggested that the gene product is a structural RNA, which may act directly on chromosomal DNA by altering the conformation of the chromatin in the region (Brockdorff et al, 1992; Brown et al, 1992). Moreover, in human females whose karyotypes include tiny ring X chromosomes were found to be unable to inactivate the tiny rings which were thus thought to be responsible for their multiple phenotypic abnormalities and severe mental retardation (Migeon et al 1993).

The specific function of *XIST* in the inactivation process is unclear (Ferguson-Smith MA, 1991). *XIST* probably plays a role in the initiation of X inactivation, although it is not required for maintenance of the inactivation state. Maintenance of X inactivation through subsequent cell division is thought to result at least in part from epigenetic changes in the genes, including methylation of CpG islands of some X-linked genes (Cattanach BM, 1975; Riggs AD, 1990), absence of acetylated histone H4 (Rastan S, 1994), chromatin condensation and allocyclic replication (Lyon 1992; Migeon BR, 1994; Disteche CM, 1995).

1.3 Sex chromosome abnormalities and chromosomal intersex

Sex chromosome abnormalities can mean many things but commonly it refers to all abnormalities of sex chromosome number and structure, and usually, as well, to discrepancies between karyotype and phenotypic sex. Chromosomal errors may be divided into: (A) Numerical changes. (B) Structural changes.

(A) Numerical chromosomal aberrations:

If a haploid gamete or a diploid cell lacks the expected number of chromosomes (n or $2n$ respectively), aneuploidy exists. When the complement contains one addition of a whole chromosome ($2n+1$), trisomy exists. The term can be applied to sex chromosomal abnormalities (e.g. 47,XXX) but if an excess number of sex chromosomes are present it is customary to use the term polysomy. If one entire chromosome is missing ($2n-1$) monosomy exists (i.e. 45,X). Polyploidy happens when more than two haploid complements are present within a single cell. Triploidy ($3n = 69$ chromosomes) and tetraploidy ($4n=92$ chromosomes) are the most common types of polyploidy. Both conditions are seen in spontaneous abortions and stillbirths and are essentially incompatible with life (Emery and Muller, 1992).

Aneuploidy results from non-disjunction in either the meiotic divisions of the parents (during gametogenesis) or in the early cleavage division of the affected individual (during embryogenesis). Aneuploidy for more than one chromosome is the result of non-disjunction in both meiotic divisions, non-disjunction in the meiosis of both parents (which must be a rare coincidence), multiple non-

disjunction in the same mitosis or meiosis, or abnormalities in more than one mitosis (Therman and Susman, 1993).

The sex chromosomes show a much wider range of viable aneuploidy than do the autosomes. Figure 1.2 summarises the non-mosaic numerical sex chromosome abnormalities found so far, their incidence in new born of the same sex, and the number of Barr bodies (all but one X chromosome in a cell is inactivated, forming X chromatin bodies, in the interphase) (Therman and Susman, 1993).

The incidence of 45,X (Turner syndrome) individuals seems to be independent of maternal age. Experimental evidence has shown that 70 to 80% of patients with 45,X retain the maternal X chromosome (Lorda-Sanchez et al, 1992; Mathur et al, 1991). On the other hand, the incidence of 47,XXX and 47,XXY individuals increases with maternal age. In these cases, non-disjunction apparently occurs mainly in maternal meiosis. Individuals with three X chromosomes (47,XXX) do not seem to form a well-defined syndrome. They are often mentally retarded and are fertile (Polani PE, 1977). Females with more than three X chromosomes (e.g. 48,XXXX) suffer from severe mental retardation and several somatic anomalies, yet their sex development is usually normal (Nielsen et al, 1977). In the group of sex chromosome abnormalities with a male phenotype, the 47,XXY and 47,XYY conditions occur approximately equal at birth. Individuals with 47,XXY karyotype form a well-defined syndrome (Klinefelter syndrome). Klinefelter syndrome is a condition in which affected men are sterile, have very small testes, sometimes have gynecomastia and may be mentally retarded (Connor and Ferguson-Smith, 1991). Men with a 47,XYY sex chromosome constitution have been reported to be higher among penal institutions for the mentally abnormal (2/1000) and in mentally deficient adult males (3/1000) (Connor and Ferguson-Smith, 1991). Males with one Y chromosome and more than five X chromosomes are mentally retarded and display various other symptoms.

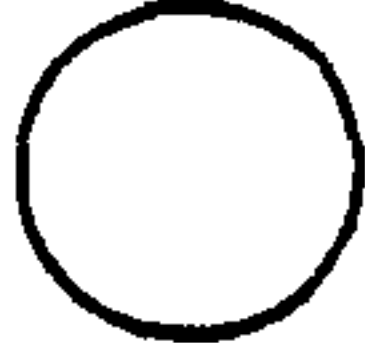




| BARR BODIES | FEMALES | MALES |
|---|--|---|
|  | <div> <div> XO 1/2000 TURNER </div> <div> XY fertile </div> </div> | <div> XYY 1/700 </div> |
|  | <div> XX </div> | <div> XYY 1/1000 KLINEFELTER </div> |
|  | <div> XXX 1/800 </div> | <div> XXXYY </div> |
|  | <div> XXXX </div> | <div> XXXXY </div> |
|  | <div> XXXXX </div> | |

Figure 1.2: The human X and Y chromosome constitutions and the Barr bodies formed by the X chromosomes (Genotypes below the broken line show mental retardation and/or mentally illness: from Therman & Susman, 1993).

(B) Structural chromosomal aberrations:

Some structural variation exists in human somatic chromosomes without apparent phenotypic consequences. However, clearly abnormal variations are usually associated with phenotypic abnormalities. Aberrations may lead to genetic imbalance through deficiency or duplication of genetic information. In deficiency or duplications only portions of a given chromosome, not the entire chromosome, are involved. However, if the variation is large enough to be recognised by light microscopy, one can assume that many genes are duplicated or deleted. Phenotypic abnormalities might also result if the position of a gene with respect to its neighbouring gene(s) is altered (position effect). Structural chromosome aberrations result from chromosomal breakage. If only one chromosome is involved, the result may be a deletion (loss of genetic material) duplication (gain of genetic material) or inversion (rearrangement of genetic material) (Fig1.3). When both ends of a chromosome are deleted followed by joining of the sticky ends, a ring chromosome is formed (Fig1.3). If the centromere divides horizontally, rather than longitudinally, separating the two chromosome arms instead of sister chromatids in normal division, an isochromosome exists (Darlington DC, 1939). An isochromosome can be composed of identical long arms {i.e. 46,X,i(Yq) }, or short arms {i.e. 46,Xi(Yp)}. If breakage involves more than one chromosome a translocation usually result (Fig1.3). In reciprocal translocations material distal to the breakage points is exchanged between homologous or non homologous chromosomes. In balanced translocation usually there is no net loss of genetic material and the individual may be perfectly normal (Brock DJH, 1993).

C) Mosaicism and chimerism

A mosaic individual has two or more genotypes (cell lines) that originate from a single zygote. Sex chromosome mosaicism is an important cause of abnormal sexual differentiation. Chromosomal mosaicism usually arises following mitotic non-disjunction, with the survival of at least two cell lines that contain different complement.

An individual with sex chromosomal mosaicism might, but not invariably, differ phenotypically from non-mosaic individuals (Hassold et al, 1980). The

differences presumably reflect the distribution of various cell lines in different tissues. A great variety of mosaics have been described in man. For example, patients with Klinefelter's syndrome have been found with the following sex chromosomes constitutions: XX/XXXY, XY/XXY, XY/XXXY, XXXY/XXXXY, and XY/XXY/XXXY. The same variety has been reported in cases with Turner syndrome (X/XX, X/XXX, X/XX/XXX, X/XYY, X/XY).

A chimeric individual has two or more genotypes each arising from a different zygote, which subsequently fuse. Dispermic chimeras are the result of fertilisation of two genetically different egg nuclei by two sperms and the resulting two diploid nuclei both contribute to the formation of the individual (46,XX/46,XY, true hermaphrodite). Blood chimeras on the other hand result from an exchange of cells between non-identical twins through the placenta while they are in the uterus (Emery and Muller, 1992).

Numerical abnormalities of human sex chromosomes are common. They occur in approximately 2-3% of conceptions (Hassold et al, 1980), and are found in 1:400 male and 1:700 female live births (Hamerton et al, 1975). Correlation of the abnormalities with the phenotype led to the conclusion that the Y chromosome contains the male determinant (Jacobs and Strung, 1959). Furthermore mosaicism of X and XY leads to dysgenesis or mental subnormality often with hypogonadism. These patients with gonadal dysgenesis and X/XY mosaicism have a 20% risk of developing gonadoblastoma (Verp and Simpson, 1987).

Short arm deletions of the X chromosome (Xp-) and long arm X isochromosome [i(Xq)] both involve loss of short arm material and both result in short stature, Turner stigmata, and gonadal dysgenesis (Ross et al, 1985; Therman and Susman, 1990). Patients with deletion of the long arm (Xq-), the critical region (Xq13-Xq26) or with short arm X isochromosomes [i(Xp)] both have loss of X chromosome long arm material and tend to have gonadal dysgenesis with normal stature and no somatic abnormalities associated with Turner syndrome (Therman and Susman, 1990; Ogata and Matuso, 1995). Other structural abnormalities such as fragments and ring X chromosome have been described (Cole et al 1980). These abnormalities are frequently associated with mosaicism including cells with X chromosome monosomy, and this makes correlation of the X chromosome constitution and phenotypic expression difficult.

X- autosome translocation has been associated with a wide range of phenotypic expression, including gonadal dysgenesis (Sarto et al 1973). The correlation of phenotypic expression and clinical manifestation, with such X-autosome translocation depends on the segment of X chromosome lost and on the arm of X chromosome involved (Ballabio and Andria, 1992; Therman et al, 1990).

Since 22 pairs of normal autosomes and at least one normal X chromosome are essential for survival of the zygote, there are no phenotypic males with X chromosome monosomy or deletion of the short arm or long arm of an X chromosome.

Structural abnormalities of the Y chromosome are rare but instructive with regard to functional segments, since Turner syndrome, intersex status, and maleness with infertility result from structural aberrations of the Y chromosome (Buhler EM, 1980; Davis RM, 1981). Short arm deletion involving the testis determining factor of the Y chromosome and Y long arm isochromosome [i(Yq)] both involve loss of the testis determining factor (Yp11.2) resulting in sex reversal (Davis, 1981). X:Y translocations involving the TDF are found in sex reversal individuals (XX males) or more rarely XY females (Petit et al 1987; 1990). Y:autosome translocation leading to loss of Yp also results in female phenotype. In the absence of a second X chromosome ambiguity of internal organs, streak gonads, or gonadoblastoma are often found (Munke et al, 1988). Structural Y chromosome abnormalities resulting in deletion of the fluorescent heterochromatin such as ring Y (Chandley, and Edmund, 1971), short arm dicentric Y (Chandely et al 1986) and other cases of Yq deletion itself are documented in infertile men (Hartung et al, 1988; Chandley et al, 1989). With regard to structural abnormalities of the Y chromosome, the most constant feature observed has been infertility.

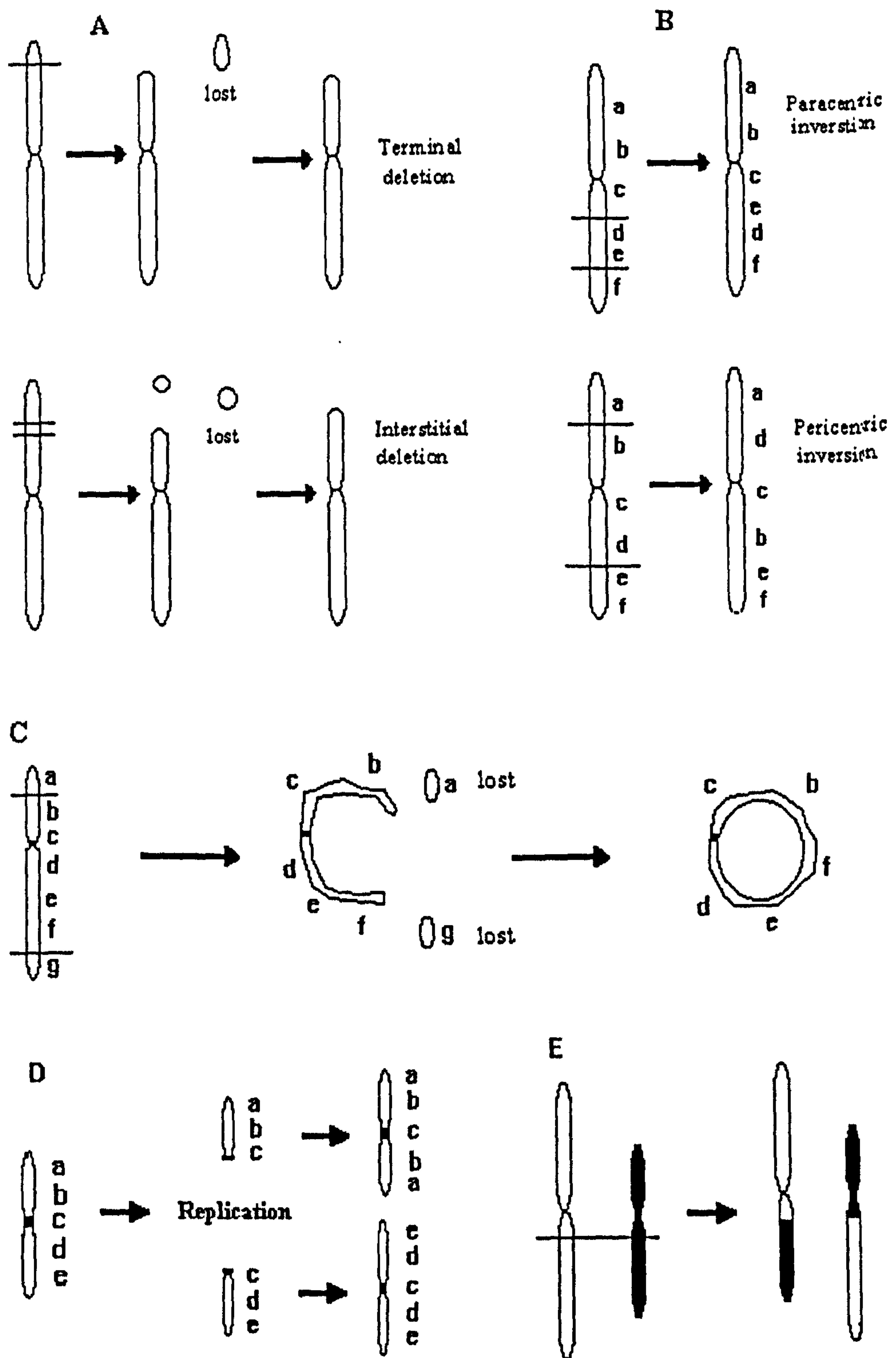


Figure 1.3: Structural chromosome abnormalities. A, Formation of terminal and interstitial deletions. B, Formation of paracentric and pericentric inversions. Break points originate from same side and on opposite sides of centromere, respectively. C, Formation of ring chromosome, D, Formation of isochromosome, E, Formation of reciprocal translocation.

1.3.1 Turner Syndrome and variants

Turner syndrome is a complex human disorder which is usually associated with a 45,X karyotype. It is the only monosomy compatible with life. It is a genetic disorder characterised by short stature, gonadal dysgenesis and various somatic stigmata (Zinn et al 1993). Overall the incidence of all abnormalities that include partial or complete monosomy X is about 1 in 2000-2500 live female birth (Nielsen and Wohler, 1991). However, it is estimated that up to 99% of Turner conceptuses are spontaneously aborted, and conversely that 5-10% of spontaneously aborted fetuses have 45,X karyotype (Hassold TJ, 1986). In contrast, the mortality rate during pregnancy for fetuses with Turner mosaic karyotype is at least 13-fold lower than for non-mosaic 45,X and this has led to the hypothesis that most if not all liveborn patients with 45,X are mosaic with a normal cell line present (Hassold et al 1988; Hook and Warburton 1983). About 50-59% of patients with Turner syndrome have a 45,X complement. Structural abnormalities of the second X [usually 45,X/46,X i(Xq)] are seen in 25% of cases. 45,X/46,XX mosaicism in 9.5%, 45,X/47,XXX in 3.5%, 45,X/46,XY in 5.5%, 45,X/46,X + mar in 3%, and more complex karyotype in the rest (Table 1.1) (Magenis et al, 1980; Rooney and Czepulkowski, 1994).

The somatic abnormalities can be classified into the following groups: (1) Anomalies attributable to lymphatic obstruction such as webbed neck, low posterior hair line, rotated auricle, puffy hands and feet, redundant skin, nail dysplasia, and whorl dominant finger pattern type (Zinn et al, 1944). (2) Skeletal anomalies, such as short neck, micrognathia, high arched palate, cubitus valgus, genu valgus, short metacarpals and metatarsals, and scoliosis. (3) Cardio-vascular and renal abnormalities such as coarctation of the aorta and horseshoe kidney. (4) Miscellaneous anomalies, such as multiple pigmented nevi (Ogata and Matsuo, 1995). The absence of secondary sexual characteristics is known to be caused by pre-pubertal ovarian failure and the consequent lack of ovarian sex steroid production. The ovaries initially develop normally, but at about six months of gestation massive oocyte loss occurs. Normal females also undergo oocyte loss in-utero, but to a lesser extent. Only small streaks of fibrous tissue remain in Turner patients. Burgoyne and Baker (1984) proposed that incomplete chromosomal pairing in meiosis might cause germ cell death.

Table 1.1: Karyotype variations in Turner syndrome.

| Karyotype | Incidence in 110 cases of TS with chromosome abnormality* | Percent |
|--|--|----------------|
| 45, X | 64 | 58.2 |
| 45,X/46,XX or 47,XXX | 11 | 10 |
| 46,X, i(Xq) including mosaics | 21 | 19.1 |
| 45,X/46,X,r(X) | 6 | 5.5 |
| 45,X/46,XY or 47,XYY | 6 | 5.5 |
| 46,X,Xp- | 1 | 0.9 |
| 46,X,t(X;autosome) | 1 | 0.9 |

***From: Rooney and Czepulkowski, 1994.**

The typical Turner phenotype is most frequently manifested by patients with 45,X karyotype. The full or partial Turner phenotype also exists in apparently non mosaic patients with various sex chromosome rearrangements (Ferguson-Smith, 1991; Grumbach and Conte, 1992). Short stature is a universal feature in 45,X and 46,X,i(Xq) or mosaics; in contrast, normal stature occurred in 20% of 45,X/46,XX; 50% of 45,X/47,XXX, and 63% of long arm deletion (Neely and Rosenfeld, 1994).

Gonadal dysfunction varied in severity among different karyotypes 45,X/46,X,i(Xq) , 46,X,idic(Xq), and 46,X,idic(Xp) were associated with severe gonadal dysfunction. In terminal X deletions, the degree of gonadal dysfunction was roughly correlated with the deleted size. Gonadal dysfunction appears to be mild in interstitial deletions of the X chromosome (Ogata and Matsuo 1995). Spontaneous pubertal development and menarche, which occurred in 8% of patients with X monosomy, were more likely in girls with 45,X/46,XX mosaicism (21%) and deletions of the short arm (25%) (Neely and Rosenfeld, 1994). There is considerable diversity, however, in reports regarding the incidence of other anomalies.

1.3.2 Hermaphrodites or intersexes

Intersexuality can be defined as the “possession at birth of both male and female characteristics in the gonads and/or in the internal or external genitalia” (Jacobs PA, 1969). In the majority of forms of intersex, there is only one type of gonad present: a testis (male pseudohermaphroditism) or an ovary (female pseudohermaphroditism). An example of the former would be testicular feminisation syndrome and for the latter, congenital adrenal hyperplasia.

Patients identified as true hermaphrodites have both testicular and ovarian tissues, although the distribution of these two gonadal tissues between the left and right sides is variable. There may be any combination of the following; ovary, testis, and ovotestis. One might expect that true hermaphrodites would be due to mosaicism (XX/XY), some of cells having an XX sex chromosome complement and others having an XY sex constitution. In fact some hermaphrodites are XX/XY mosaicism (Josso et al, 1965; Jacobs PA, 1972), but for some reason which is still not clear the sex chromosome constitution of many of these patients

appears to be 46,XX (60%). 46,XY constitution has been found in 15% of these patients (Hunter RF, 1995). Of course hermaphroditism in the presence of an XX karyotype contradicts the requirement for a Y chromosome (or TDF) to enable differentiation of testicular tissue. Possible explanations for the presence of testicular tissue in 46,XX hermaphrodites include: (1) undetected loss of a Y chromosome after initiation of testicular development, or undetected chromosomal mosaicism such as X/XY or XX/XXY or XX/XY chimaerism. (2) The translocation of TDF from the Y to an X chromosome or to an autosome. (3) A single gene mutation (Eicher and Washburn, 1983).

Since the availability of suitable cytogenetic techniques in the late 1950s a large variety of sex chromosomal abnormalities have been documented in man. Many but not all of these, have been shown to be associated with different degrees of abnormalities of primary and secondary sex character. The most frequently reported chromosome abnormality associated with intersexual development is mosaicism, where one cell line has 45,X constitution and the other has 46,XY or 45,X/47,XXY sex chromosome constitution (Jacobs PA, 1969). The phenotype of such individuals will depend on the time after fertilisation when the error giving rise to the two cell lines happened, and the frequency and distribution of the two types of cells in the embryonic gonad. In fact, almost every degree of intersexual development has been found to be associated with an X/XY constitution (Hunter RF, 1995). Another type of mosaicism expressed as intersex condition is when one cell line is that of a normal female while the other has a Y chromosome (XX/XXY or XX/XY/XXY) (De La Chapell A, 1972). An intersex phenotype may also arise from an XX/XY chromosome complement (Jacobs PA, 1969; 1972).

1.4 The mammalian sex-determining master regulator gene SRY (sex determining region on the Y chromosome)

1.4.1 XX and XY sex reversal

One of the more elegant lines of evidence for testis determination being effected through the Y chromosome comes from analysis of sex reversal individuals, viz. 46,XX males, XX true hermaphrodites, and XY females. About 1 in 20,000 newborn males have a 46,XX karyotype and yet develop testes (McElreavey et al, 1993b). These individuals are sterile with small testes and

azoospermia. Two phenotypically distinct groups have been recognised among subjects with 46,XX sex reversal. 46,XX maleness is characterised by the presence of testes in a subject with a female karyotype. Most affected individuals have normal male external genitalia, but about 10% have hypospadias (De La Chapell, 1972).

46,XX true hermaphrodites are characterised by the development of ovarian and testicular tissue in the same individual, either in separate gonads or in one gonad (Ovotestis) [Van Niekerk and Retief, 1981; Torres et al, 1996]. These patients usually present with ambiguous external genitalia whereas the development of Wolffian and Mullerian duct derivative depend on the type of gonad and extent of testicular development in each case (Berkovetz et al, 1992; Perez-Placios et al 1994). In 1966, Ferguson-Smith proposed that 46,XX maleness and 46,XX true hermaphrodites are generated by rare and illegitimate X:Y chromosomal interchange during paternal meiosis when the X and Y chromosomes pair, enabling Y-specific sequences to be transferred to the short arm of the paternal X (Fig1.4). The Y-specific transferred sequences were considered to include the testis determining factor (TDF) giving rise to either complete (XX male) or partial (XX true hermaphrodite) masculinization. Two lines of evidence confirmed this hypothesis. First, cytogenetic analysis of XX males revealed in some cases that the distal G-bands of the short arm of Y (Yp11.3) are translocated onto the distal end of the short of the paternal X chromosome (Evans et al, 1979; Magenis et al, 1987). Second, molecular studies showed that about 80% of XX males and 10% of XX true hermaphrodites carry Y sequences on the tip of the short arm of their paternal-derived X chromosome (Abbas et al, 1990; McElreavey et al, 1992a).

Human XY females with pure gonadal dysgenesis (also known as Swyer syndrome) have a 46,XY karyotype and no sexual ambiguity. Approximately 1 in 100,000 newborn females are XY females (Polani PE, 1981). This syndrome is defined by the presence of female genitalia, normal Mullerian structures, streak gonads and the absence of testes which result in lack of puberty, primary amenorrhea and high levels of gonadotropins (Marcantonio et al, 1994). The female phenotype together with an apparent absence of testicular tissue implies a mutation in the initial steps of male sex determining pathway. In contrast to XX males some XY females have deletions of portions of the Y chromosome. Since

the amount of Y material whether absent (XY female) or present (XX male) is variable, this allowed a precise genetic map of the Y chromosome to be constructed (Vergnaud et al, 1986) and led to precise mapping of TDF to the short segment of Y chromosome adjacent to the pseudo-autosomal boundary (Affara et al, 1986; 1987; Vergnaud et al, 1986).

1.4.2 The search for the sex determination gene(s)

For more than 10 years, many investigators believed that the male-specific H-Y histocompatibility antigen was the product of TDF (Wachtel et al, 1975). However, the discovery of male mice failing to express H-Y excluded (in mouse) this gene as a possible candidate (McLaren et al, 1984). By chromosome walking between the break points present in an XX male and an XY female, Page et al (1987) have isolated a second candidate for TDF. They identified a zinc finger protein on the Y chromosome (ZFY) that resembles a group of transcription factors that bind to *cis* acting DNA elements and regulates gene transcription, which is compatible with regulatory switches such as TDF, and therefore ZFY was thought to be a candidate for TDF. However, its autosomal location in marsupials (Sinclair et al, 1988), its inappropriate expression in mouse tissue (Koopman et al, 1989) and finally its location outside minimum sex determining region of the human Y (Palmer et al, 1989) excluded it from this function.

Using the same strategy that identified ZFY, Sinclair et al (1990) and Gubby et al (1990) isolated *SRY* (sex determining region on the Y chromosome), a single copy gene, which meets the criteria for TDF. It is located in the smallest Y chromosome region capable of causing sex reversal. It is expressed for a short time in the genital ridge (when testicular cords form) during embryonic development (Koopman et al, 1990) and is deleted or mutated in some cases of human XY females (Berta et al 1990; Jager et al 1990; Hawkin et al, 1992). Finally, this conclusion was confirmed by the male development of XX mice that were made transgenic for mouse *Sry* (Koopman et al, 1991).

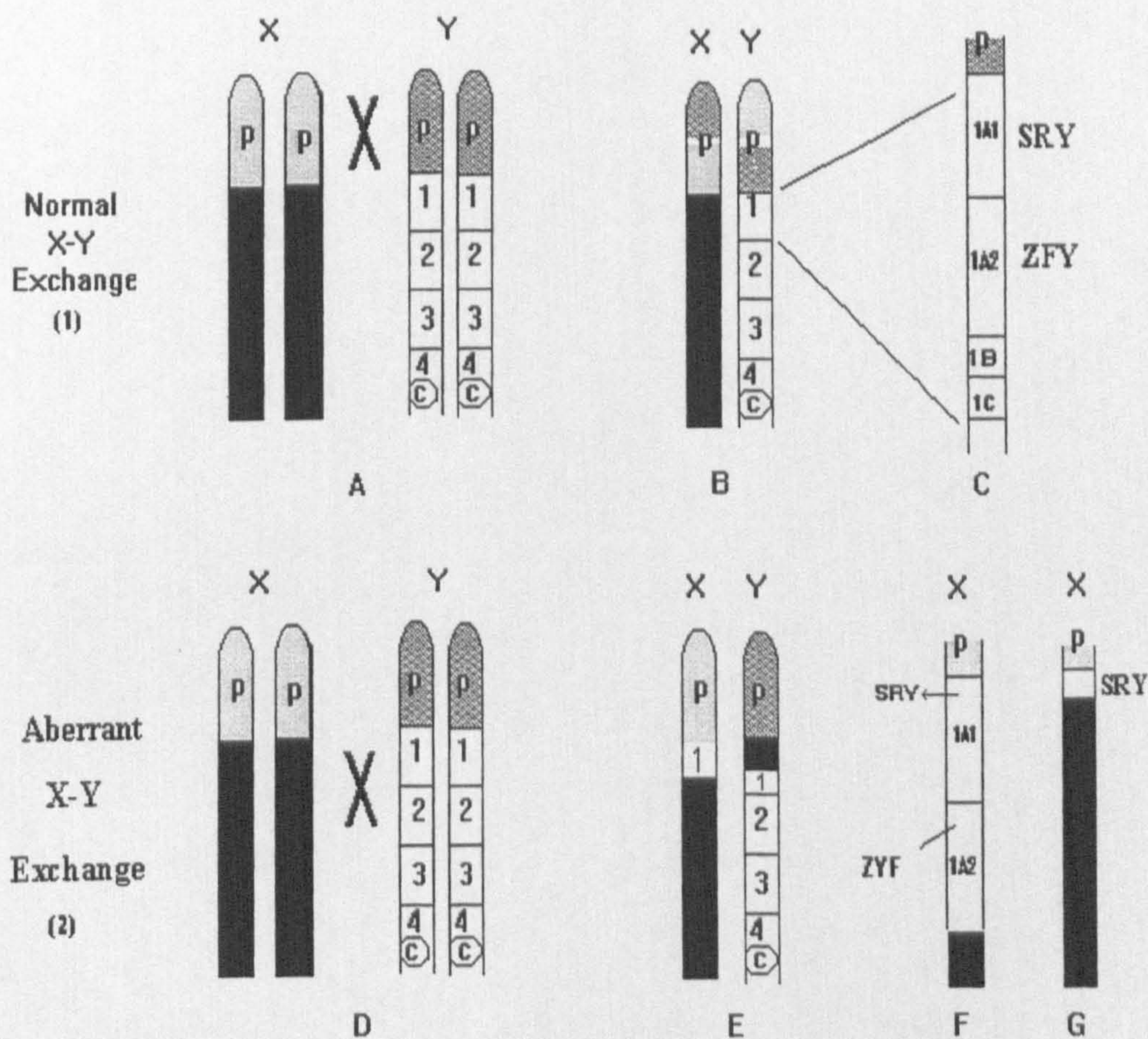


Figure 1.4: A close-up view of the region of exchange between the X and Y chromosomes. (1) Indicates the manner of normal exchange between the pseudoautosomal regions [PAR] (P) as indicated in A-C. (2) Illustrates the manner of accidental interchange of testis-determining region or factor (TDF) outside the so-called pairing region from the Y to X chromosome enabling an X chromosome to carry *SRY* during errors in the paternal meiosis generating XX males (F-G). The deleted Y chromosome (*SRY* negative) generates XY females (E) (from Burgoyne, 1992).

1.4.3 The *SRY* gene

The *SRY* gene as mentioned above, was identified by positional cloning within the region of the Y chromosome critical for male sexual development (Gubby et al 1990; Sinclair et al, 1990). It is an intronless gene potentially coding for a protein with 204 amino acids of which 79 show homology to the HMG (High Mobility Group) box, similar to nuclear chromosomal non histone protein HMG1 and HMG2, which bind DNA (Su and Lau, 1993). The HMG-box is very conserved in a wide variety of mammals; conversely, the protein segments flanking the HMG-box are highly variable in sequence and length (Bianchi et al, 1992; Grosschedl et al, 1994). Human *SRY* and mouse *Sry* proteins are 89% homologous in the HMG-box region but outside this diverge significantly (Tucker et al 1993; Whitfield et al 1993; Pontiggia et al 1995). The *SRY* cDNA sequences correspond to a direct copy of the genomic DNA with transcript size ~1.1 kb on Northern blot (Sinclair 1990; Su and Lau, 1993) and 612 bp translated (Behlke et al, 1993). Sequencing a cDNA clone isolated from adult testis identified the 3' end of the transcript is mapping to position 747 from the first ATG (Methionine) codon of the open reading frame (ORF). The 5' end of *SRY* transcript is difficult to analysis and the results from different groups are contradictory. Vilain et al (1992) defined the transcription initiation site at 91 bp upstream from the first Methionine, while Su and Lau (1993) defined two transcription start sites at position 78 bp and 136 bp. The reason for these different results is not clear but it may be due to the different tissue tested (Goodfellow and Lovell-Badge, 1993). Moreover, the *SRY* transcriptional unit is immediately preceded by two small GC-rich segments covering part of the 5' untranslated region and sequence immediately 5' of the *SRY* gene. Several binding sites for the transcription factor SP1 are identified among these GC-rich sequences. The 5' flanking region lacks an obvious TATA or CAAT box within the GC-rich sequences immediately upstream of the transcription start sites. However, several partial sequences (e.g. TATA, CATA, GATA, and TACA) homologous to the TATA box are present within this region, their exact role as promoter is unknown (Su and Lau, 1993).

The function of *SRY* is still not completely clear. The *SRY* protein has been shown to bind specifically to DNA (Harley et al, 1992b) and the DNA binding

properties are entirely determined by the HMG-box (Bianchi et al, 1992; Grosschedl et al, 1994). Gieze et al (1992) showed the SRY protein binds to the minor groove of the double helix and recognizes a higher affinity binding site defined by the nucleotide sequences 5'-CCATTGTTCT and produces a sharp bend, bringing distinct regions of the genome into juxtaposition and possibly enhancing protein-protein contact. Alternatively by bending the DNA, *SRY* could inhibit the binding of other transcription factors and therefore could play the role of a repressor, preventing the female sex determining genes, thereby effecting transcriptional control (Soullier et al, 1994). The HGM-box was found also to interact with high affinity to kinked DNA structures irrespective of their sequences (Ferrari et al, 1992). However, the importance of this binding for *SRY* function is not known. Furthermore, point mutations associated with sex reversal in XY human females fall within the HMG-box (Berta et al 1990; Hawkins et al 1992; Jager et al 1992). And it was found that these mutations either affect the affinity of DNA or modify the geometry of the DNA- protein complex (Pontiggia et al, 1994; 1995).

In XY females with gonadal dysgenesis, mutations have been identified and so far some 23 different mutations are known, accounting for 10-20% of cases (Wolf et al, 1992; Schmitt-Ney et al, 1995). Thus in the majority of patients with XY gonadal dysgenesis (80%) the *SRY* does not appear to be affected and in some XX males the *SRY* gene is not present, pointing to the existence of other sex determining gene(s) (Goodfellow and Lovell-Badge, 1993; McElreavey et al, 1993a, b; Poulat et al, 1994).

The mutations within the *SRY* gene identified so far are with two exceptions clustered in the HMG-box region, indicating that this is the functional domain of the *SRY* protein. The exceptions are a case with a deletion 5' to the gene which may affect *SRY* expression (McElreavey et al, 1992b) and two sibs with an identical stop codon mutation 3' to the HMG-box (Tajima et al, 1994). Five of these mutations cause termination codons (causing truncated protein) and the others are small deletion or point mutations (Goodfellow and Lovell-Badge, 1993; McElreavey et al, 1992b; Schmitt-Ney et al, 1995). Four familial cases of XY sex reversal have been described where the affected individuals share the same amino acid changes as their father and sometimes as other male relatives (Harley et al, 1992b; Jager et al, 1992; Vilian et al, 1992; Schmitt-Ney et al, 1995).

Familial mutations are considered to be conditional, their manifestations depend on the genetic background and epigenetic or environmental interaction. Thus it has been suggested that for *SRY* to fulfil its biological function bending of DNA at the site of its action must be geometrically precise (Pontiggia et al, 1994).

In the XY sex-reversal females only the HMG-box regions have been analysed extensively for mutations therefore, it is possible that undetected mutations, lie elsewhere in the *SRY*.

1.4.4 Genes that interact with SRY

The evidence that *SRY* is the master testis determining switch is compelling, but many observations show that sex determination is more complicated than a simple, single switch (Moore and Grumbach, 1992). *SRY* is assumed to act on transcription only indirectly by promoting the association of transcription factors, resulting in their activation. Therefore, there are likely to be many other genes involved in mammalian sex determination upstream and downstream of *SRY*. There is no evidence that any of these lie on the Y chromosome. Several autosomal or X-linked genes participating in the pathway have been proposed and reported (McElreavey et al, 1993a,b; Bogan and Pade, 1994; Wolf U, 1995).

SRY gene is thought to influence gonadal morphogenesis into male pattern of dispersed support cells surrounding germ cells in seminiferous tubules. It interacts with as yet unknown *SRY* intermediate factor(s) (*SRYIF*) to induce the *MIS* gene, which in turn causes regression of the female Mullerian duct derivatives. *SRYIF* may be one of several factors participating with *SRY* in the determining of gonadal sex. Sterogenic factor 1 (SF-1) is one of the *SRYIF* candidates that play a role in *MIS* regulation. *SRY* may also activate other target genes on autosomes such as SRA-1 (autosomal sex reversal factor). The dosage sensitive sex reversal locus (DSS) on the X chromosome may also act by repressing the *SRY*-induced male pathway or by activating expression of genes required for formation of female structure (Fig 1.5) (Haqq et al, 1994).

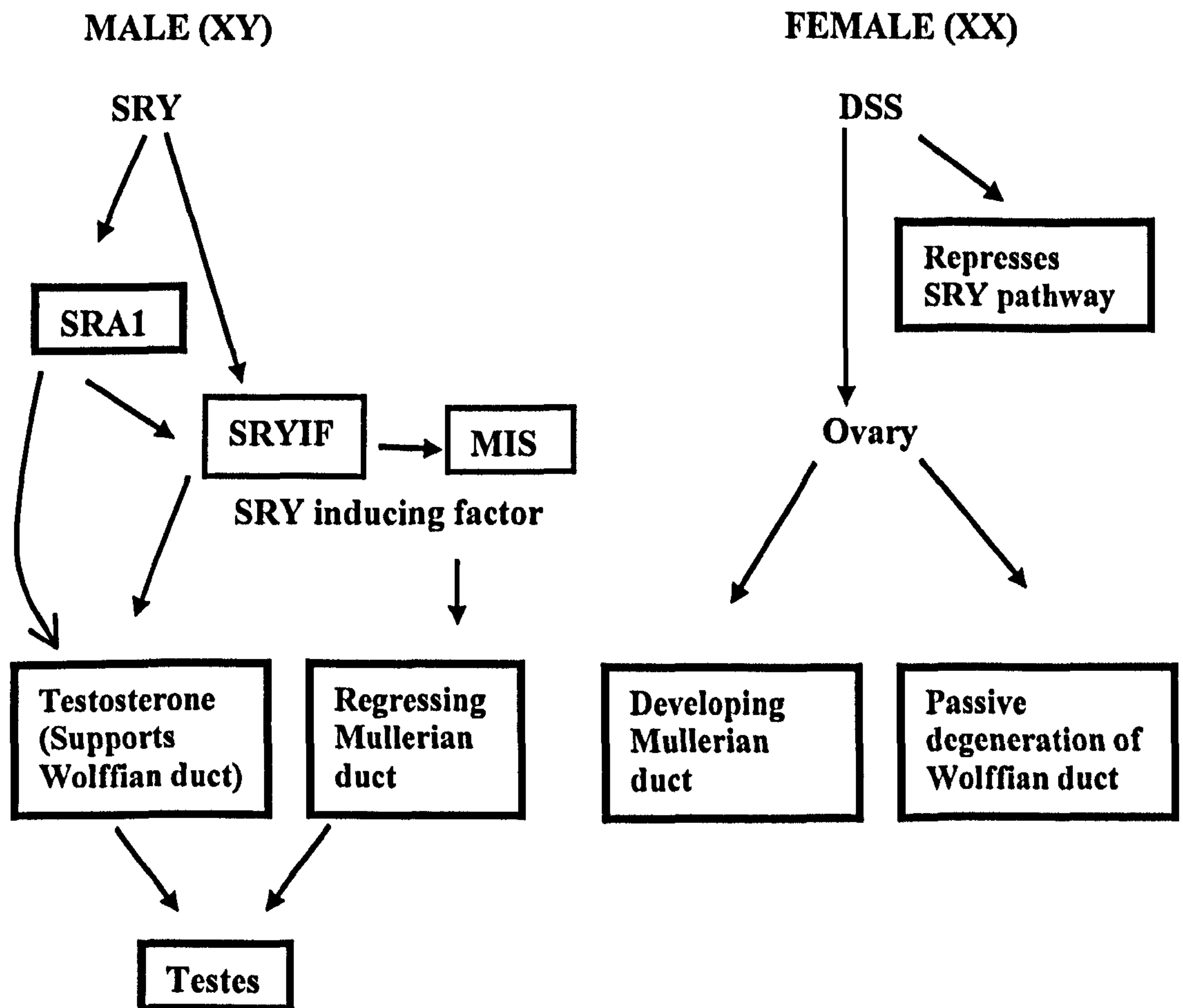


Figure 1.5: A hypothetical scheme for sex determination in mammals. The male regulatory pathway is initiated by SRY. A factor or factors (SRYIFs) mediates induction of the MIS gene by SRY. SRYIF may interact directly with the MIS promoter, or indirectly through modification of the basal transcription machinery. MIS, in turn, plays a key role in male sexual development as a diffusible substance causing regression of the female Mullerian duct derivatives: uterus, fallopian tubes, and vagina. SRY may activate other genes on autosomes such as SRA1. The DSS may repress the SRY-induced male pathway or by activating expression of genes required for formation of female structures (modified from Haqq et al 1994).

(A) Autosomal genes

(1) Wilm's tumour gene (WTI):

In man, one of the genes frequently considered to be involved in gonadal development and differentiation is WTI, the Wilm's tumour suppressor gene at 11p13 (Call et al, 1990; Van Heyningen and Hastie, 1992). Wilm's tumours are autosomal dominant childhood kidney cancers that affect 1 in 1000 children (Mastunga E, 1981). Some patients with Wilm's tumour also have aniridia, genitourinary abnormalities and mental retardation (WAGR syndrome), and others have Denys-Drash syndrome (nephropathy, genital abnormalities and predisposition to the development of Wilm's tumour). In both clinical conditions there is malformation of the gonads resulting in either ambiguous genitalia or complete sex reversal without genital ambiguities (McElreavey et al, 1993b). The *WTI* gene encodes a zinc finger transcription factor expressed in tissues that develop into kidney and gonad (Pelletier et al, 1991; Pritchard-Jones et al, 1990).

Heterozygous point mutations in the *WTI* gene have been observed in Denys-Drash syndrome. And the XY individuals who are heterozygous for certain mutations in *WTI* with Drash syndrome, the gonads showed varying degree of dysgenesis and the phenotypes ranged from normal female to a male with hypospadias with or without cryptorchidism (Pelletier et al, 1991; Baird et al, 1992; Bruening et al, 1992).

The role of *WTI* in normal gonad formation is still unclear. A mouse model for studying WTI function has been developed by introducing a mutation in the mouse *WTI* gene (Kreidberg et al, 1993). All fetuses homozygous for *WTI* null allele showed a phenotype similar to Denys-Drash syndrome with streak gonads. Gonadal development is arrested at a very early stage, consistent with the earliest expression of *WTI* observed in wild-type animals at 9 dpc (days post coitum), which is before the time of primary gonad differentiation. Thus it is thought that *WTI* is required for early commitment of gonadal tissue and the establishment of the bipotential gonad (Kreidberg et al, 1993). Also it has been speculated that *WTI* may act upstream of *SRY* and sex determination and that targets include genes other than *SRY* (Bogan and Pade, 1994).

(2) Mullerian inhibiting substances (MIS):

MIS is thought to be one of the candidate genes that operate down stream of the *SRY* gene (Bogan and Pade, 1994; Haqq et al, 1994). The *MIS* gene was

cloned in 1986 and mapped to the short arm of chromosome 19 (19p13.3) (Picard et al, 1986; Cohen-Haguenauer et al, 1987; Cate and Wilson, 1993). It belongs to a superfamily of growth factors important in cell growth, differentiation and regulation. The family includes transforming growth factor β , inhibin, activin and bone morphogenetic factors. Apart from *SRY*, *MIS* is the first biochemical marker of differentiation and is secreted by Sertoli cells. Even though *MIS* acts downstream of *SRY* in sex differentiation it is not obvious that *MIS* function in gonadal determination is strictly defined. In persistent Mullerian duct syndrome (PDMD), a form of male pseudohermaphroditism, patients have Mullerian duct structures and unilateral or bilateral undescended testes, but normal testicular differentiation. Certain individuals with PDMD syndrome harbour *de novo* mutations in *MIS* (Carre-Eusebe et al, 1992). The lack of effect of these mutations on testicular organogenesis is against *MIS* as a testicular differentiation factor (Bogan and Pade, 1994).

The early expression of *MIS* in testicular differentiation raises the possibility that the *MIS* gene is regulated by *SRY* directly. Several observations suggest that it is not. Evidence has recently been provided that *MIS* expression occurs by interaction of SF-1 with the promoter region of *MIS* gene, and indeed in Sertoli's cells the spatiotemporal expression of *MIS* and SF-1 is concordant during embryonic development (Shen et al, 1994). *In situ* hybridisation experiment in mice first detected *Mis* expression at 12.5 dpc in Sertoli cells, approximately 48 hours after the onset of *Sry* transcription (Munsterbeg and Lovell-Badge, 1991). This observation (delayed onset of *Mis* expression) raises the possibility that other steps are involved. *In vitro* studies of *MIS* expression demonstrated that *MIS* transcription could be induced by *SRY* even if the binding of *SRY* to *MIS* promoter is abolished by mutation (Haqq et al, 1994). Therefore, *MIS* induction by *SRY* is thought to be indirect and intervening *SRY*-induced factors (*SRYIF*) are proposed which transduce the *SRY* signal to the *MIS* promoter. A candidate for such factor is SF-1. SF-1 can bind to a *MIS* regulatory element (*MIS-RE-1*) and in the presence of *SRY*, the transcription of SF-1 is upregulated, while in its absence (in female) SF-1 transcription decreases (Shen et al, 1994). Moreover, SF1 has been shown either alone or combined with *SRY* to decrease the *MIS* expression (Haqq et al, 1994), indicating that SF-1 acts as a transcriptional repressor. The interaction of SF-1 with the *MIS* gene seems to be established. However, its interaction with *SRY*

appears to be more indirect because of the temporal difference in peak expression of SRY and SF-1 during testis differentiation (Wolf U, 1995).

(3) Autosomal sex reversal:

An autosomal XY sex reversal locus SRA1 associated with campomelic dysplasia has been mapped to the distal long arm of chromosome 17 at 17q24.3-q25.1 (Tommerup et al, 1993). An *SRY*-related gene *SOX9* (*SRY*-related HMG box) was isolated and found to encode a transcription factor expressed in many adult tissues, in foetal testes and skeletal tissues (Foster et al 1994; Wagner et al, 1994). Furthermore, mutations in *SOX9* gene demonstrated that this gene is responsible for both campomelic dysplasia (severe skeletal abnormalities in both XX and XY individuals) and varying degrees of sex reversal in XY individuals only, including intersexual and female phenotype, and gonadal dysgenesis or even ovarian like histology (Houston et al, 1983; Foster et al, 1994; Wagner et al, 1994). From the pleiotropic effect it has been speculated that *SOX9* has a function in both skeletal and gonadal development (Foster et al, 1994). The gonadal phenotype in campomelic dysplasia could result from mutation of a gene involved in forming the bipotential gonad (like WT) or involved in gonadal sex determination (like SRY) (Bogan and Pade, 1994).

(B) X-linked genes

There are two genes on the X chromosome which if mutated suppress male development of XY foetuses: the androgen receptor (AR) and dosage sensitive (DSS) loci.

Dosage Sensitive Sex reversal locus (DSS):

The existence of an X-specific gene involved in human sex determination was postulated after observations of families with apparent X-linked modes of inheritance of 46,XY gonadal dysgenesis (Fechner et al, 1993). Furthermore, evidence was provided by the identification of sex reversal patients carrying duplications of portions of the Xp. The fact that two active copies of chromosomal region Xp21.1-22.1 can cause sex reversal was initially reported by Arn et al (1994) and was referred to the sex reversal X locus (SRVX). Shortly after, Bardoni and colleagues (1994) confined the sex reversal region to Xp21 and named it the dosage sensitive sex reversal (*DSS*) locus (Bajalica et al, 1995). To date twenty

cases with partial duplications of the X short arm and an intact SRY gene has been described (Bajalica et al, 1995). XY individuals with an X carrying the duplication show various forms of intersexuality and sex reversal ranging from incomplete testicular differentiation to streak gonads. On the other hand XY individuals with deletions of the DSS region develop as male, while females are not affected by heterozygous duplications within this region. Therefore, Bardoni et al (1994) concluded that the DSS gene is not involved in testicular differentiation, but a double dose of DSS protein interferes with this process. They speculated that DSS plays a part in the ovarian pathway and that in the presence of *SRY* a single dosage of DSS can be overcome, but not a double dosage of it (Bardoni et al, 1994; Wolf U, 1995). Thus it is thought that it may act either by repressing the SRY-induced male pathway or by activating the expression of genes required for formation of female structure (Haqq et al, 1994).

1.5 Androgen Receptor (AR) and Molecular Genetics of Androgen Insensitivity Syndrome (AIS)

Androgens [testosterone (T) and 5 α -dihydrotestosterone (DHT)] are essential for sexual differentiation of the male embryo and for the regulation of virilization in the adult. Androgens, secreted by foetal testes are responsible for the initial growth and differentiation of the male reproductive tract, including the Wolffian ducts, the urogenital sinus, and the primordial of the external genitalia (Fig 1.6). During puberty, androgens promote the appearance of secondary male sex characteristics, including growth of the external genitalia, development of the prostate and seminal vesicles, male distribution of body hair, and increase in total muscle mass. These hormones are essential for initiation and maintenance of spermatogenesis and they have a negative feed back control on the secretion of gonadotropins by the hypothalamic-pituitary axis (Brown TR, 1995).

Testosterone, secreted by the testes, is responsible for virilization of the Wolffian ducts. Development of the prostate and external genitalia are dependent upon the tissue-specific conversion of testosterone to its more biologically potent DHT by 5 α -reductase (Griffin JE, 1992). Testosterone and DHT mediate their function through the intracellular androgen receptor (AR) which belongs to the steroid, thyroid hormone and retinoic acid receptor family (Evans RM, 1988).

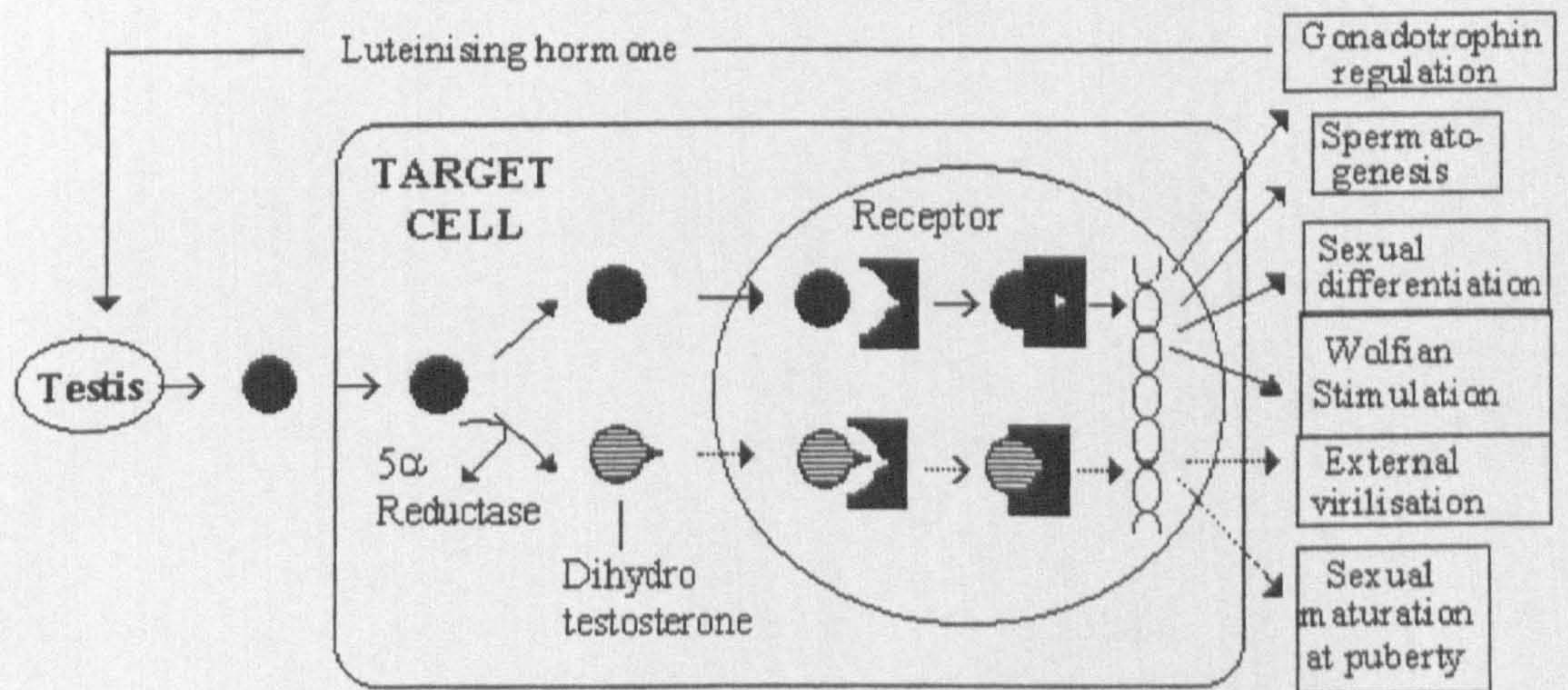


Figure 1.6: A model of a target cell response to androgens, the major actions of which are listed to the right of the cell. Testosterone enters the cell and binds either to the androgen receptor in the nucleus or is first converted enzymatically to dihydrotestosterone: action of testosterone is indicated by continuous arrows, that of dihydrotestosterone by broken arrows (From Wilson, 1992).

1.5.1 Androgen receptor (AR) structure and function

The AR gene locus spans over 90 kb and has been mapped to the long arm of the X chromosome q11-12 region (Brown et al, 1989; Kuiper et al, 1989). The human AR gene is a single copy gene consists of 8 exons (Lubahn et al, 1989). The 5' untranslated region (5'UTR) encoded by exon 1, is 1.1 kb in length and contains one major and one minor site of transcription initiation in a 13 bp region. The promoter lacks TATA and CCAAT like elements, but has GC rich sequences including a putative SP-1 binding motif at -50 and -60 (Tilley et al, 1990; Faber et al, 1991). The 3'UTR, encoded by exon 8, is 6.8 kb long, and is postulated to play a role in the stability and/or translation of AR mRNA (Mizokami and Chang, 1994). There are two poly (A)⁺ signals. Northern blot analysis of RNA showed the presence of a predominant 10 or 11 kb mRNA, with a less abundant, alternatively processed 7 or 8 kb mRNA (Faber et al, 1991). The 110-114 kb receptor protein contains 910-919 amino acids encoded by a 2.75 kb open reading frame (Chang et al, 1988; Tilley et al, 1989). Studies of the steroid receptors including human AR, have revealed the presence of three conserved functional domains (Evans RM, 1988).

(i) The transactivating N-terminal domain:

The amino terminus is the least conserved region with the greater variation in length among steroid receptors, but is important for transcriptional activation of target gene by the human AR (Jenster et al, 1991). It contains 529-539 amino acids and is encoded by exon 1. A distinguishing feature of the DNA sequence encoding the N-terminal domain is the presence of two polymorphic trimeric repeats of (GGN)_n and (CAG)_n (Trifiro et al 1994). The GGN repeats (16-27 mer) encoding glycine stretch, start at codon 449 and the (CAG)_n repeats (15-31 mer) encoding a polyglutamine stretch, start at codon 189 (Jenster et al, 1991). The polymorphic variation within these segments accounts for the variable number of amino acids (910-919) in the human AR protein between individuals.

(ii) The DNA-binding domain:

The central domain, encoded by exons 2 and 3, contains 66 amino acids. This cystein-rich region is the most highly conserved region within the steroid receptor and is important for DNA-binding to target DNA (Lubahn et al, 1989). It

shares approximately 80% homology with the same domain of the glucocorticoid (GR) and progesterone (PR) receptors. By structural analogy to GR (Hard et al, 1990; Luisi et al, 1991), the DNA-binding domain consists of two zinc fingers which each of them being tetrahedrally co-ordinated to four cysteine (Evans RM, 1988; Sultan et al, 1993). Crystallography studies on the GR have indicated that the first zinc-finger (N-terminal) specifies DNA recognition of the receptor, while the second finger is mainly responsible for dimerisation of two receptor molecules during their association of DNA (Fawell et al, 1990; Guiocho-Mantel et al, 1989). This DNA-binding domain interacts with *cis*-acting elements of the regulated genes [androgen responsive element (ARE)], which are 15-nucleotide-long palindromic sequences located in the flanking region of the gene (Sultan et al, 1993; Ham et al, 1988).

iii) The steroid-binding domain:

The carboxy terminus (C-terminal) of AR (250 amino acids) is encoded by exons 4-8 and is responsible for ligand (steroid) binding affinity and specificity (Jenster et al, 1991) and in the interaction with heat-shock proteins such as HSP90 (Veldscholte et al, 1992). The binding of the two physiologically active androgens (Testosterone and DHT) to the receptor coincides with the dissociation of heat shock proteins and conformational activation of the hormone-receptor complex (Brown TR, 1995).

The actions of androgens on target cells occur via the classical steroid receptor pathway. The action is initiated by the interaction of the steroid with the androgen expressed in target tissue. Androgen binding induces a process of receptor activation involving dissociation from receptor-associated proteins (Smith and Toft, 1993). The activated receptor binds as a dimer (Wong et al, 1993) to a specific DNA sequences (ARE) in the promoter regions of target genes and modulates transcription of those genes (De Bellis et al, 1994).

1.5.2 AIS and genetic abnormalities of the AR gene

Androgen insensitivity syndrome is a rare-X linked disorder (1/60,000 male birth) caused by an inability to express the androgen effect in 46,XY individuals with testes able to synthesise androgen. This impaired response to androgen result from the inability or reduced ability of the androgen receptor (AR)

to transactivate androgen responsive genes in target cells, and leads to abnormal differentiation and development of internal and external genitalia in male, and thus leads to male pseudohermaphroditism (Griffin JE, 1992). Depending on the extent of AR defect, the phenotype varies and usually the complete form [Complete AIS] (CAIS)] is distinguishable from the partial ones [partial AIS (PAIS)]. In CAIS, primarily referred to as testicular feminisation, affected individuals are genetic males presenting with female external genitalia, without ambiguity, however, the vagina is short and blind. They are usually diagnosed in the neonatal period because of inguinal hernia, or at puberty because of primary amenorrhea associated with normal breast development and absent axillary and pubic hair. Female internal genital structures are usually absent and testicular histology show spermatogenesis to be incomplete or absent, although Leydig cells are abundant. Plasma testosterone concentrations are within normal to elevated as a result of the increased stimulation by luteinising hormone (LH). In contrast, PAIS is heterogeneous and associated with a wide spectrum of clinical disorder from males with severe ambiguous genitalia (i.e. Cryptorchidism, micropenis and hypospadias) to a phenotypic male with severe hypospadias (Reifenstein syndrome) (Pinsky et al, 1992; Ris-Stalpers et al, 1994), to unexplained male infertility (Aiman and Griffin, 1982).

The clinical heterogeneity of AIS is matched by a diversity of biochemical abnormalities of the AR receptor. The first indication that the AR itself is defective in AIS came from androgen studies on genital skin fibroblast cell line from AIS patient (Keenan et al, 1974; Hughes et al, 1986). These defects can be grouped into four broad categories: (a) Negative binding (AR-), (b) qualitative defects in receptor function (thermal instability of the protein), (c) deficient amount of apparently normal receptor (AR), and (d) receptor-binding positive (AR+), a category in which the endocrine, phenotype, and genetic features are compatible with a receptor abnormalities but no functional abnormalities been identified (McPhaul et al, 1991; McPhaul et al, 1993). The majority of CAIS patients are receptor negative. In contrast, no clear cut relationship between clinical phenotype and AR binding exist in PAIS patients; the majority of cases show normal receptor binding, although a small group show qualitative abnormalities in the AR binding.

Recently, cloning of the AR gene led to molecular genetic studies to identify lesions in the AR gene. When binding is negative or qualitatively

abnormal, mutations are identified in the exons encoding the ligand binding domain of the AR gene, whereas normal AR binding in CAIS is associated with mutations affecting the DNA binding region (Griffin and Wilson, 1992; McPhaul et al, 1993). A number of mutations of the AR gene have now been reported in both CAIS and PAIS. Complete or partial AR gene deletions have been found in 5-10% of all AR gene mutation causing AIS (Trifiro et al, 1991; Quigley et al, 1992a, b; Brown TR, 1995). Deletions involving multiple (4-8, or 3-8, and 6-7) and single exon (2, 3, or 5) have been reported for families with CAIS (Brown et al, 1994). Deletion of part of intron 2 and 3 were reported in only one case with PAIS (Ris-Stalpers et al, 1992) and deletion of exon 4 was detected in a patient with only azoospermia (Aiken et al, 1991).

The vast majority of mutation in both CAIS and PAIS are point mutations in the coding region of the AR gene causing amino acid substitutions (Brown et al, 1990; Maracelli et al, 1991; McPhaul et al, 1991), or premature termination (Sai et al, 1990; Trifiro et al, 1991; De Bellis et al, 1992), or aberrant mRNA splicing (Ris-Stalpers et al, 1990; Brown TR, 1995). Most of these substitutions were localised in three regions of the AR: the DNA binding domain and 2 segments of the ligand binding domain. Two hot spots for mutations are present in the AR gene namely exon 5 and exon 7 (McPhaul et al, 1993; Sultan et al, 1993).

The evidence to date indicates that mutation of the AR gene is responsible for most cases of CAIS. In contrast, many patients with PAIS demonstrate no defect in androgen binding and no AR gene mutation can be identified (Batch et al, 1992; McPhaul et al, 1993). Moreover, an attempt to assign a phenotype to a particular mutation have failed (Griffin JE, 1992; McPhaul et al, 1993). The same mutation has been reported to result in different phenotypes (McPhaul et al, 1991; McPhaul et al, 1993). The reasons for such discrepancies between patients exhibiting the same molecular defect are still poorly understood.

1.5.3 AR gene mutations in other diseases

It is interesting to note that AR gene mutation is also involved in other diseases. In a study of 26 patients with prostate cancer, only one somatic point mutation in the ligand-binding domain (exon 5) has been described in a patient with early stage cancer (Newmark et al, 1992). The authors postulated that this

mutation might be involved in the development or progression of prostate cancer. Moreover, Wooster et al (1992) reported a germ line mutation responsible for an Arg-Gln substitution at position 607 in two brothers with breast cancer and PAIS. Lobaccaro et al (1993) reported a substitution of position 608 in one out of 13 male patients with breast cancer. Both mutations occurred in the DNA binding domain (in exon 3). Lobaccaro et al (1993) postulated two hypotheses that may explain the development of breast cancer linked to AR mutations. One possible explanation is that the mutated AR gained an ability to bind to oestrogen responsive element (ERE), thus activating oestrogen regulated gene. Although this region of the binding was never shown to be involved in the binding specificity to the response elements, yet the authors claimed a 'tri-dimensional' structure modification might play a role. The second hypothesis is loss of the protective effect of the androgen. Thomas et al (1992) suggested that the breast cancer in men develop in response to androgen deficiency and under conditions associated with excess oestrogen. Lastly, La Spada et al (1992) showed that spinal bulbar muscle atrophy (SBMA or Kennedy syndrome) is due to the expansion of the normal polyglutamine tract (Tandem CAG repeat) from 15-31 to 40-50. This adult onset neurodegenerative disorder is often associated with mild androgen insensitivity (gynecomastia, testicular atrophy, and infertility). SMA has never been reported in AIS with any other AR mutation. It was postulated that this expansion of the polyglutamine region could cause structural constraint of the N-terminal domain resulting in modification of transcription initiation complexes on target genes (Brown TR, 1995).

1.6 Male Infertility

The end process of sex determination and differentiation is the ability to reproduce. Approximately 15-20% of couples have difficulty or are unable to conceive (Purvis and Christiansen, 1992; Bhasin et al, 1994) and in one-third of cases it is the male who is considered the predominant reason for the infertility (Bhasin et al, 1994). Most authorities define these patients as prematurely infertile if they have been unable to achieve a pregnancy after twelve months of regular intercourse without contraception (Greenhall and Vessy, 1990; te Velde and Beets, 1992).

1.6.1 Spermatogenesis and its hormonal control

Spermatogenesis is a complex developmental process. The progression from spermatogonial stem cells to mature spermatozoa requires 65 days and involves elaborate succession of distinct cell types. The process is carried out by at least three mitotic and two meiotic divisions (Reijo et al, 1995). The primitive cells (spermatogonia), either divide to reproduce themselves for stem cells renewal or they divide to produce daughter cells that will later become spermatocytes. The spermatocytes then divide and give rise to mature cell lines that eventually give rise to spermatids. The spermatids then undergo a transformation into spermatozoa (Dym M, 1994). Spermatogenesis begins at puberty and continues throughout adult-life. A human male may produce 10^{12} to 10^{13} gametes during his life time (Reijo et al, 1995).

Spermatogenesis requires the stimulatory effects of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and adequate testosterone (T) concentrations (Matsumoto and Bremner, 1989). FSH is enough to be required for maturation of spermatids but its role in maintenance of spermatogenesis is less clear (Soham et al, 1994). Luteinizing hormone is thought to have effect on initiation and maintenance of spermatogenesis indirectly in that it stimulates androgenous testosterone production (Purvis and Hansson, 1978).

1.6.2 Causes of male infertility

Male infertility can result from a number of different changes within the male genital tract but generally infertile men can be divided into five major diagnostic categories (Cummins et al, 1994). (1) Disorders related to motility or function of the sperm such as congenital defect of the sperm tail, sperm maturation defects and immunologic defect (sperm antibodies) (Purvis and Christiansen, 1992; Cummins et al, 1994). (2) Disorders related to obstructive lesions (obstructive defect) which most commonly affect the exocrine ducts of the testes but may also involve the more distal duct system such as the ejaculatory ducts themselves (Cummin et al, 1994). Around 7-15% of all patients attending infertility clinic was shown to have obstructive lesions (Dubin and Amelar, 1971). (3) Disorders related to spermatogenetic failure as a result of lesion in the testis itself has been reported to

be present in ~30% of infertile men (Jaquier and Holmes, 1993). (4) Sexual dysfunction disorders of erection and ejaculation. (5) Endocrine dysfunction. Patients with endocrinopathy such as hypogonadotropic hypogonadism, are rarely seen in the infertility clinic, with an incidence of approximately 3% (Purvis and Christiansen, 1992).

Environmental factors, genetic factors or a combination of both may cause defects in male reproductive function. Environmental factors with known effects on reproductive function include nutrition (obesity or undernutrition), infection, excessive physical exercise, toxic substances, radiation, smoking and excessive consumption of coffee or alcohol (Negro-Vilar A, 1993; Fauser and Hsueh, 1995). Recent studies have begun to focus on genetic defects underlying disturbed human male reproductive function.

1.6.3 The genetics of male infertility

The genetic disorders related to male infertility can be divided into three categories: (i) pre-testicular, (ii) testicular and (iii) post-testicular causes of male infertility (Mak and Jarvi, 1996). These genetic defects are responsible for a variety of clinical presentations of male infertility, from gonadotropin-releasing hormone deficiency such as congenital hypogonadotropic syndromes (Kallmann's, Prader-Willi and Bardet-Biedl syndromes), to spermatogenic failure such as chromosomal abnormalities (Klinefelter, XX males and deletion of the azoospermia factor gene), to obstructive azoospermia such as mutation in the cystic fibrosis transmembrane regulatory gene (*CFTR*) (Table 1.2).

Table 1.2: Summary of genetic disorders related to male infertility

i) Pre-testicular:

| DISORDER | CLINICAL FEATURES | GENETIC MECHANISM |
|--|---|---|
| Kallmann's syndrome | Anosmia, Craniofacial asymmetry, cleft palate, delayed puberty, small testes | X-linked recessive, variable inheritance; defect in KALIG-1 gene on Xp22.3 chromosome |
| Prader-Willi syndrome | Obesity, mental retardation, hypotonia; small hands and feet | Often due to interstitial deletion of chromosome 15q12 (paternally inherited) |
| Bardt-Biedl syndrome | Obesity, mental retardation, Retinitis pigmentosa, polydactyly | Autosomal recessive; gene mapped to chromosome 16q21 |
| Cerebellar ataxia with Hypogonadotropic hypogonadism | Speech delay, gait, lack of secondary sexual characteristics, eunuchoidism, decreased libido, small firm testes | High incidence of parental consanguinity, Autosomal recessive inheritance |
| Sickle cell anemia | Anemia, sickling of erythrocytes at low oxygen tension | Autosomal recessive inheritance, defect in gene for β -globin chain of hemoglobin |
| β -Thalassemia | Anemia, iron overload | Autosomal dominant inheritance, defect in gene for β -globin chain of hemoglobin |

ii) Testicular :

| | | |
|-------------------------------------|--|---|
| Klinefelter's syndrome | Tall stature, azoospermia, extragonadal cell tumors, hypogonadism, small firm testes, diminished secondary sexual characteristics. | 90% of cases 47,XXY karyotype. Remainder are mosaics for polysomy for X or Y chromosome |
| XYY | Tall stature, may exhibit aggressive behaviour. | Karyotype 47,XYY |
| Deletion of azoospermia factor gene | Normal | <i>De novo</i> deletion of azoospermia factor gene mapped to Yq11.22-23 chromosome |
| 46,XX male | Phenotypically masculine Small firm testes | 46,XX, SRY gene present, azoospermia factor gene absent |
| Noonan's syndrome | Short stature, hypertelorism, webbed neck, low set ears, cubitus valgus, ptosis, pulmonary stenosis, cardiac anomalies, cryptorchidism, testicular atrophy | 46,XY, autosomal dominant inheritance |
| Myotonic dystrophy | Myotonia, muscle wasting, cataract, frontal balding, electrocardiographic changes, testicular atrophy | Autosomal dominant inheritance, with variable penetrance, expanded number of CTG sequence repeats in gene mapped to 19q13.3 |

iii) Post-testicular :

| DISORDER | CLINICAL FEATURES | GENETIC MECHANISM |
|---|--|---|
| Cystic fibrosis | Chronic respiratory infections, pancreatic insufficiency, elevated sweat chloride, wolffian duct derivatives atrophic or absent. | Autosomal recessive inheritance, defect in CFTR gene on chromosome 7q31.1 |
| Congenital bilateral absence of vas deferens | Wolffian duct derivative atrophic or absent, otherwise healthy | Autosomal recessive inheritance, defect in CFTR gene in patients without concomitant renal anomalies |
| Idiopathic epididymal obstruction Young's syndrome | Obstructed epididymis, otherwise healthy Chronic sinopulmonary infections, obstructed epididymis | Defect in CFTR gene implicated Autosomal recessive inheritance, defect in CFTR gene implicated |
| Polycystic kidney disease | Multiple cysts in kidney, liver, spleen, pancreas, lung, ovary, testes, epididymis, seminal vesicle, cerebral aneurysms, mitral valve prolapse, colonic diverticulosis | Autosomal dominant inheritance, 2 genes implicated (PKD1 on 16p13.3 and PKD2 on 4q chromosomes) |
| Immotile cilia syndrome | Chronic retinitis and sinusitis, nasal polyposis, bronchiectasis, immotile spermatozoa | Autosomal recessive inheritance, genetic heterogeneity |
| 5- α -reductase deficiency | Perineoscrotal hypospadiasis, ventral urethral groove, blind vaginal pouch, female habitus without breast development | Autosomal recessive inheritance, gene encoding 5- α -reductase iso-enzymes 1 and 2 located on chromosome 5p15 and 2p23 |
| Androgen insensitivity syndrome | Complete /incomplete testicular feminization, Reifenstein's syndrome, infertile male syndrome | 46,XY, X-linked recessive inheritance, defect in androgen receptor gene located on Xq11-12 chromosome |

*Modified from: Mak and Jarvi, 1996.

1.6.3.1 Chromosomal abnormalities as a cause of male infertility:

(1) Numerical chromosome abnormalities:

Klinefelter syndrome is a genetic disorder due to the presence of an extra X chromosome in the male, the common karyotype is either 47,XXY (classic form) in 90% of cases or 46,XY/47,XXY (the mosaic form) in 10% of cases. This disorder is a common genetic cause of male infertility, with an incidence of 1/1000 male births (Connor and Ferguson-Smith, 1991). More than 30 karyotypic varieties of Klinefelter syndrome has been described such as, XXYY, XXXY, XXXXY, and various mosaicisms of X chromosome with or without associated structural abnormalities of the X chromosome (Griffin and Wilson, 1992). Characteristically, these individuals have small, firm testes, delayed sexual maturation, azoospermia and gynecomastia. Testicular biopsy often reveals sclerosis and hyalinization of the seminiferous tubules, absence of spermatogenesis and increased Leydig cells. LH and FSH levels are characteristically elevated. Testosterone levels can range from normal to low and decrease with age.

The mosaic variant of Klinefelter syndrome (46,XY/47, XXY) is usually not as severe as the classic form and fertility has been documented in some patients with mosaicism (Foss et al, 1971). The testes may be normal in size and gynecomastia and azoospermia are less common. The frequency of the mosaic form may be underestimated, since chromosomal mosaicism may occur only in the testes while karyotypes on peripheral leukocytes may be normal (Griffin and Wilson, 1992).

The incidence of the XYY syndrome (1per 1000 male birth) (Connor and Furguson-Smith, 1991) is the same as that of Klinefelter syndrome but its phenotype expression is more variable. These patients are excessively tall and 1-2% has anti-social behaviour. Semen from these patients may vary from azoospermia to normal. Testicular biopsy specimens show maturation arrest to complete germinal aplasia. Most have a normal LH and testosterone level with the FSH level dependent on the extent of germ cell damage.

(2) Structural chromosome abnormalities: Structural Y chromosome disorders include 46,XX male karyotype and deletion of the azoospermia factor gene.

XX disorder or sex reversal syndrome: it is a variant of Klinefelter syndrome. Male individuals with this disorder are phenotypically masculine but have a 46,XX karyotype. The signs are similar except for the average height which is less than normal, hypospadias is common and decreased incidence of mental deficiency. These patients have a 46,XX chromosome complement with an incidence of 1/20000 males (McElearvey et al 1992a). It is postulated that in these patients, the sex determining region of the Y chromosome (*SRY* gene), which encodes the putative testis determining factor, is present, however the azoospermia factor (AZF) is missing in the genome. The mechanism of this disorder thought to be due to aberrant X-Y interchange at paternal meiosis which may occur because of the proximity of TDF (*SRY*) to the pseudo-autosomal region where recombination between X and Y chromosomes normally occurs (Page et al, 1987).

1.6.3.2 Deletion of the azoospermia factor (AZF)

Several genes are likely to be involved in the complex process of spermatogenesis. There is increasing evidences for the presence of such gene(s) in the distal long arm of the Y chromosome. Cytogenetic studies in sterile men with deletions of the long arm and other structural abnormalities of the Y chromosome have been observed in men with azoospermia and suggested the localisation of a gene controlling spermatogenesis, named azoospermia factor (AZF), resides in Yq11.23 (Tieplo and Zuffardi, 1976; Chandley et al, 1989). Molecular mapping localised AZF to interval 6 of the Y chromosome (Anderson et al, 1988) as defined by Vergnaud et al (1986). As a first step towards the molecular isolation of AZF, its gene structure in Yq11 has been mapped using two interstitial deletions in the proximal and distal part of Yq11 (Ma et al, 1992; Vogt et al, 1992a, b) and a detailed map of interval 6 was constructed (Ma et al 1992). Based on these non overlapping deletions, Vogt et al (1992a) postulated that there may be two spermatogenesis genes in distal Yq11, AZFA and AFZB, or a very large gene similar to the Y fertility gene of *Drosophila*. Deletion of the azoospermia factor accounts for 10% of 46,XY men with non-obstructive azoospermia (Johnson et al, 1989; Vogt et al, 1992a,b; Nagafuchi et al, 1993). It was estimated that at least

1/10,000 male newborn carry a *de novo* deletion of the AZF (Vogt et al 1992a). Histological studies of testicular biopsies from azoospermic men with deletion in AZF region exhibited a wide spectrum of spermatogenetic defects from complete absence of germ cells (Sertoli-cell-only syndrome) to maturation arrest with occasional production of mature, condensed spermatids (Tiepolo and Zuffardi, 1976; Chandley et al, 1986; Chandley et al, 1989; Vogt et al, 1992a,b).

In 1993, the finding of overlapping interstitial Yq deletion in 3 azoospermic men led to identification of *YRRM1* and *YRRM2* (Y chromosome ribonucleic acid recognition motif), now renamed as *RBM1* and *RBM2* (RNA binding motif) (Chandley and Cooke, 1994). It was proposed that absence of these closely related genes might be the cause of azoospermia (Ma et al, 1993).

1.6.3.2.2 Isolation of RBM gene family

The *RBM* (*YRRM*) gene family were originally cloned, on the basis of its position, with probes deleted in a small number of infertile men with phenotypes ranging from oligospermia to a complete absence of germ cells (Ma et al, 1993). Two *RBM* gene copies were isolated as cDNA clones (MK5, MK29). The 1878 bp MK5 cDNA shows the AUG starting codon is present at 118 bp, followed by an open reading frame of 1491 bp which predicts a novel peptide of 469 residue. The 1874 bp MK29 cDNA displays 7 nucleotide substitution and a 5 bp deletion relative to MK5 causes a translation frame shift that truncates the MK29 open reading frame, giving a predicted peptide of 419 residue. Both clones contain a consensus poly-adenylation signal, and MK29 contains a short poly (A)⁺ tail (Ma et al, 1993).

1.6.3.2.3 RBM protein contains an RNA-binding-related motif

The predicted peptide sequence showed 5' homology to a super family of RNA binding protein (HnRNPs) that contains a 90 amino acids RNA recognition motifs (RRM), often in a tandemly repeated array (Kenan et al, 1991; Ma et al, 1993). The *RBM* genes were found to have similarity (60% amino acid conservation) to HnRNPG (human glycosylated nuclear RNA-binding protein) (Chandley and Cooke, 1994). The majority of the genes code for protein which

have two copies of an RNA binding domain (RRM) but a subset has been found whose members, like RBM genes and HnRNPG, have only a single domain. A feature of the RRM domain is a pair of very highly conserved motifs, an octapeptide RNP1 and a hexapeptide RNP2. These parts of the protein probably form the beta sheet, which contact the RNA directly (Ma et al, 1993; Chandley and Cooke, 1994). Because of the RNA recognition motif and because of their Y chromosome localisation these genes were called *RBM1* (MK5) and *RBM2* (MK29). These RRM motifs showed the highest similarities to the third RRM repeat in mouse polyadenylate-binding protein (PABP) (Adam et al, 1986; Sach et al, 1986). Adjacent to RRM domain is a 139 residue followed by four repeats of a 37 residue (111bp), all of which showed high conservation at both the nucleotide and predicted protein level. The repeated domain is rich in serine, arginine, glycine, and tyrosine content, with no aliphatic leucine, isoleucine, and methionine and valine residue. Adjacent to the tetrapeptide SRGY box (Serine-Arginine-Glycine-Tyrosine) is a carboxyl tail of 122 amino acid in *RBM1* and 45 amino acid in the *RBM2* gene due to the deletion that is present beyond the SRGY box (Ma et al, 1993).

Expression studies showed that *RBM* sequences are confined to germ cells (testis specific) and the genes showed a male-specific conservation of expression in DNA from several other mammals (Ma et al, 1993; Chandley and Cooke, 1994). Moreover, interphase *in situ* hybridisation and Southern blot studies showed that the *RBM* sequences compose a multigene family clustered in 1-2 Mb regions of the Y chromosome with majority of the genes in intervals 5 and 6 of Yq11.23. The *RBM* gene family has approximately 30 genes and pseudogenes, one or some of which encode HnRNPG-like protein expressed in adult testis (Prosser et al, 1996). The HnRNPG proteins are widely expressed proteins and these proteins are abundant in the nucleolus as histones (Soulard et al, 1993). Ma et al (1993) have reported that the *RBM* gene family may be involved in RNA trafficking between nucleus and cytoplasm and implicated in RNA process. Therefore, they have suggested that the *RBM* gene family may have a role in spermatogenesis and are candidates for AZF. Transcription of the *RBM* genes has not been detected outside the testis, distinguishing them from HnRNPG (Chandley and Coole, 1994).

1.6.3.2.4 RBM deletions in oligo and azoospermic patients

Ma et al (1993) detected deletion of *RBM* (*YRRM*) sequences in two infertile (oligo and azoospermic) patients with no previous deleted mutations. Moreover, Kaboyashi et al (1994) analysed DNA from 63 Japanese men with either azoospermia or severe oligospermia whose Y chromosomes were cytogenetically normal. They examined 15 loci included *RBM1* locus on the long arm of the Y chromosome. They detected microdeletion in 10 of the infertile men. The *RBM1* gene was involved in only 3 of them. They proposed that the *RBM1* (*YRRM1*) is not the only gene essential for spermatogenesis.

1.6.3.2.5 RBM1 gene structure

Recently Prosser et al (1996) characterised the exon/intron boundaries of the *RBM1* gene. Twelve exons were defined. Exons 1-11 consist of 100 bp each (88-117 bp) and the 12th was slightly larger (<600 bp). Introns in the repeated SRGY box region of the 3' half of the gene are equal in size (450 bp) and each repeat is in a separate exon (Exon 7-11). The remaining introns, 1-6 and 11 varied from about 400 bp to 3 kb in length. The RRM motif extends from exon 2-4, with the highly conserved sequences RBP1 and RBP2 in exons 3 and 2 respectively. The 1.8 kb *RBM1* transcript covers a genomic region of 15kb.

1.6.3.2.6 New candidate gene of AZF

Using 84 sequence-tagged-site (STS) markers covering the 30-megabase euchromatic region, Reijo et al (1995) looked for Y chromosome deletions in 89 azoospermic males who had no signs of any physical obstruction and showed that the *RBM1* and *RBM2* genes were present in all infertile men studied, including 12 patients with interstitial Yq deletion of the azoospermia factor region. In 12 of these patients (13%), they found overlapping *de novo* deletions spanning a common region of about 500 kb in the distal segment of the Y long arm. They identified a novel gene within this region, which they named "deleted in azoospermia" (DAZ). Like *RBM*, *DAZ* is expressed specifically in the testis and

contains an RNA recognition motif, however, unlike *RBM*, *DAZ* is a single-copy gene in humans and chimpanzees. These results suggest that *DAZ* is the candidate gene for AZF and not *RBM1* and *RBM2*.

At least three different AZF genes are present on the long arm of the human Y chromosome in Yq11. They are named AZFa, AZFb, AZFc (Vogt P, 1995; Vogt et al, 1995). The positions of these genes were marked according to the results of the search for microdeletion in Yq11 (Henegariu et al, 1994). They appeared as *de novo* mutations in males with severe oligospermia and azoospermia (Vogt et al, 1995). Deletions in AZFa or AZFb disrupt the germ cell development in males before the proliferation phase of spermatogonia or at meiosis, respectively, while deletion in AZFc region disrupts the germ cells development during the maturation phase of spermatids or spermatozoa. Patients with AZFc deletion can display different amount of mature sperm cell (Vogt P, 1995; Vogt et al, 1995). Candidate genes for expression of AZFb are copies of the *RBM* gene family. Candidate genes for expression of AZFc are *DAZ* and *SPGY1* (spermatogenesis gene on the Y). Candidate genes for expression of AZFa are not yet known (Vogt et al, 1996).

A variety of other chromosome abnormalities involving the sex chromosome and/or autosomes associated with male sterility have been documented (Chandley and Edmund, 1971; Gabriel-Robez et al, 1987; Speed and Chandley, 1990; Mohandas et al, 1992). Although definitive proof of a causal relationship between these chromosomal abnormalities and spermatogenic abnormalities till now is lacking, however, these reports suggest that genes within such chromosomal regions may have a role in spermatogenesis. At least three different mechanisms resulting in abnormal spermatogenesis have been postulated (Maraschio et al, 1994). (1) Microdeletions that could affect azoospermia factor involved in spermatogenesis (Ma et al, 1992). (2) Alteration of the correct sequence order of the Y chromosome interfering with the steric organisation of the sex vesicle (Gonzales et al, 1981; Rivera and Diaz-Castanos, 1992) and 3) Autosomal interference with the X-Y bivalent (Chandley AC, 1981) through interference with X-inactivation at meiotic prophase (Forjt J, 1982), a prerequisite for normal spermatogenesis (Lifschytz and Lindsley, 1972).

1.7 Mutation detection methods

A wide variety of techniques are available for detecting disease-causing mutation within human genes. These methods are used to determine whether a candidate gene is causally related to a phenotype and also to identify new alleles at a known locus for diagnostic, population genetics and structural function studies.

The spectrum of disease causing mutation ranges from cytogenetically visible chromosome rearrangements (such as large deletion, insertion, inversion, or duplication) to microdeletion, duplication and insertion and finally single base changes. Some candidate genes, such as Dystrophin are prone to deletions (Chamberlain et al, 1989) while others such as *CFTR* gene in cystic fibrosis patients are associated with hundreds of point mutations (Tsui LC, 1992). Some genes can be as complex in structure as dystrophin, with a 14 kilobase (kb) peptide coding region and 80 exons (Koenig et al, 1988) or as simple as the *SRY* (Sinclair et al, 1990) locus with only one large exon. Moreover, many genes of interest may not be expressed in easily accessible samples such as blood, as in male infertility *RBM* gene family (Ma et al, 1993), leaving analysis of genomic DNA as the only option. Therefore, the most applicable screening method is influenced by the expected nature of the mutation, size and structure of the gene in question, availability of mRNA, degree of sensitivity required and resources available (Grompe M, 1993).

In general, the methods for mutation detection can be divided into two distinct groups. The first consists of techniques, which identify human disease alleles, and the second group consists of methods of screening for unknown mutations (Figure 1.7).

1.7.1 Screening for unknown mutations

Techniques have been devised to permit rapid screening for mutations in cases where the common mutations are not known. Each technique has its advantages and disadvantages for a given situation.

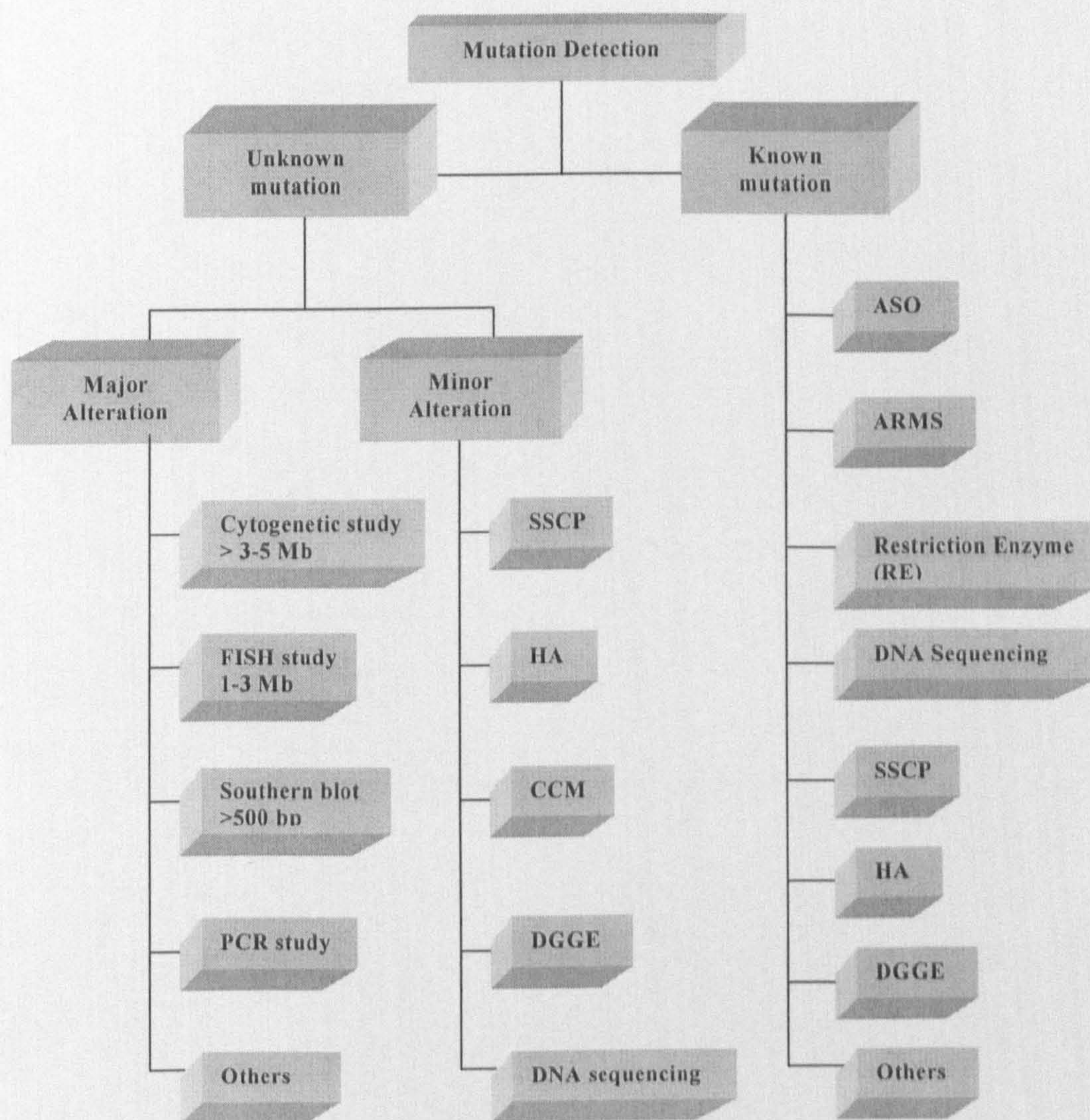


Figure 1.7: Schematic representation of mutation detection strategies.

1.7.1.1 Detection of large gene alteration

Methods for identifying mutations that grossly alter a gene (>500 bp portion) are well established. Standard and specialised cytogenetic techniques can really identify mutations that significantly disturb chromosome structure. Techniques that assess genomic organisation, including pulsed-field gel electrophoresis, Southern blot hybridisation and multiplex polymerase chain reaction (PCR) can be used to identify large deletions, insertions, inversions, or duplications.

(A) Cytogenetic techniques

Cytogenetic analysis is one of the most common approaches to genetic testing. It is the study of genetic material at the cellular level. Routine laboratory human cytogenetics is concerned with light microscope studies of chromosomes. Almost all human cytogenetic studies involve the examination of a dividing cell population requiring preliminary cell culture followed by blocking cell division at metaphase with an inhibitor of spindle formation.

There are many different staining techniques. The Giemsa (G) banding method is the most widely used for routine karyotyping (Seabright M, 1971; Sumner et al, 1971). This can resolve 400-500 bands (alternating light and dark bands) per haploid karyotype. At this level of resolution, deletion of the order of 5-10 Mb can be visualised using routine G-banding. Using high resolution level of banding (prometaphase) deletions as small as 2-5 Mb can be detected (Yunis J, 1976). This technique has led to the identification of an increasing number of small deletions causing specific syndromes such as del (11p13) for aniridia/Wilms tumour, del (15q13) for Prader-Willi and Angelman syndromes and others (Ballabio A, 1991). The chromosome banding pattern obtained is thought to reflect both the structural and functional composition of the chromosomes (Holmquist et al, 1982). Dark bands contain adenine and thymine (A+T) rich DNA, appear to contain relatively few active genes and generally replicate their DNA late in S-phase (Sumner AT, 1982). A similar banding pattern can be obtained by staining

with quinacrine (Q) and visualising with fluorescent microscope (Caspersson et al, 1970). Polymorphic variations in: (1) the centromeric region of chromosomes 1, 9, 16, (2) the satellites region of the acrocentric chromosomes, (3) and variation of the heterochromatic region of the Y chromosome (Yqh) can often be recognised using the Q-banding method. Reverse banding in which the bands stain in opposite pattern from the G-bands is used routinely in some laboratories. This technique may be useful if the telomeres are involved in aberration (Dutrillaux et al, 1973). Special techniques for staining the highly repetitive DNA in heterochromatin at the centromeres especially of chromosomes 1, 9, and 16 and at the distal region of the long arm of the Y chromosome (C-banding) (Arrighi and Hsu, 1971) are usually applied to investigate chromosome rearrangement near centromeres and to confirm polymorphism. On the other hand, the nucleolar organiser region (NOR) using the silver stain, can be applied to selectively identify chromosome containing genes for ribosomal RNA (rRNA) which are located on the short arm of acrocentric chromosomes (13, 14, 15, 21, 22) (Bloom and Goodpasture, 1976). Furthermore, the differential replication staining, produced by incorporation 5-bromodeoxy uridine (BrdU) into chromosomes, is applied to the detection of different cycles of replication within a single cell cycle (Rooney and Czepulkowski, 1992). Initially the main technique for such studies involved feeding cells with a radioactive chromosome constituent and the results were analysed with autoradiography (Taylor JH, 1958). Recent methods however, are based on the introduction methods that detect the incorporation of Brdu (thymidine analogue) into the chromosome. Chromosomes and chromatids that have incorporated Brdu, instead of thymidine, have a different structure and consequently different staining preparation from those containing thymidine. Substitution with Brdu has also been successfully demonstrated in the late-labelling X chromosome (late replicating X chromosome) and other individual chromosomes and segments that replicate in the later part of the S period (Latt SA, 1973; 1974).

(B) Molecular cytogenetic (Fluorescence *in situ* hybridisation)

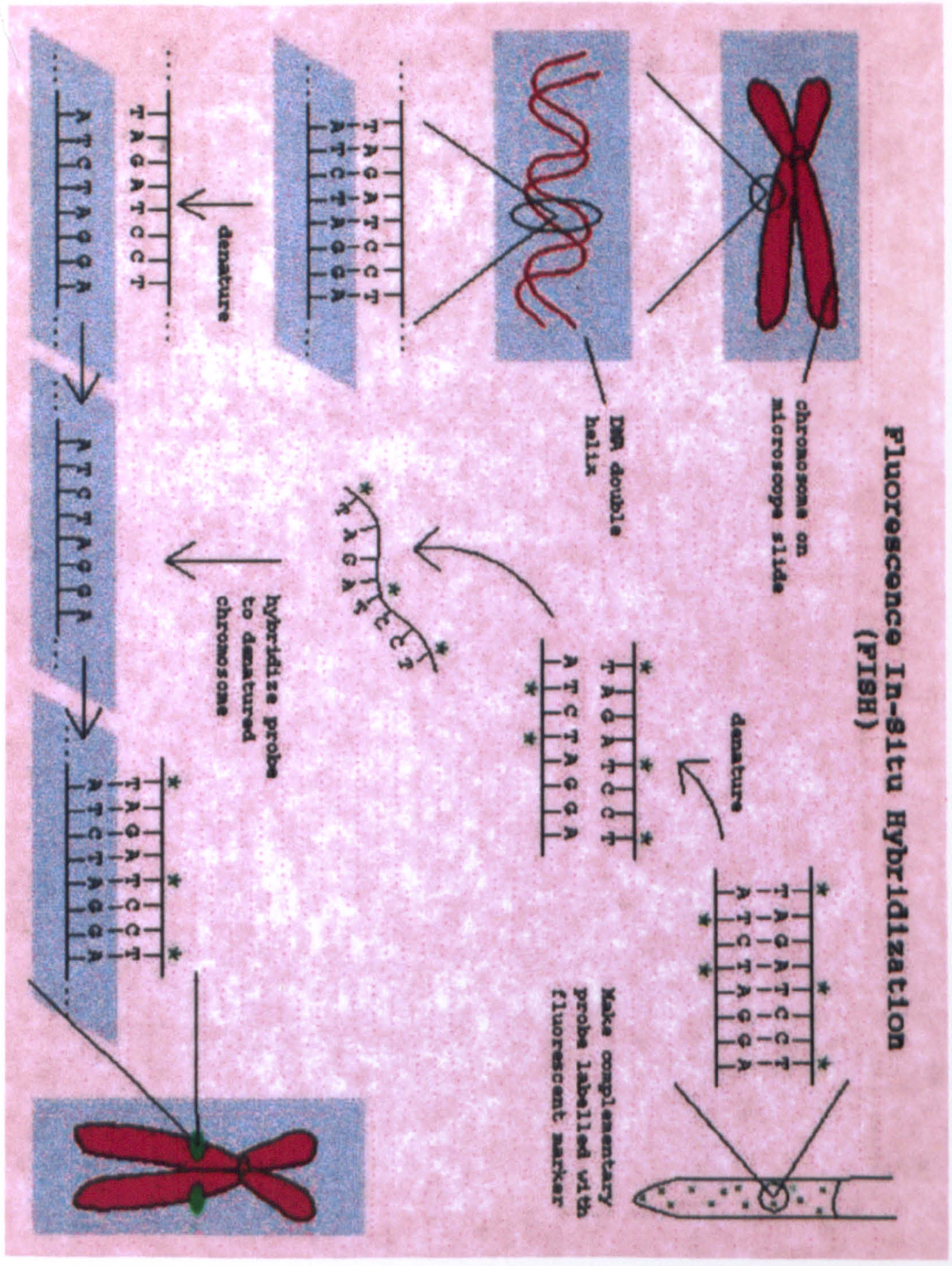
Recently, the power of cytogenetic analysis has been enhanced by the use of fluorescence *in situ* hybridisation (FISH) (McNeil et al, 1991; Tkachuk et al, 1991; Lichter and Cremer, 1992). It has been used as an adjunct to routine

cytogenetics to achieve a higher sensitivity and better resolution of chromosome aberrations as compared to banding analysis. Although chromosome analysis by conventional methods is very precise and gives a comprehensive view of the 23 pairs of chromosomes, it is time consuming and requires highly skilled cytogeneticists.

FISH has become a powerful tool both of basic science and diagnostic applications. In basic science, FISH has strongly enhanced gene and genome analysis enabling rapid mapping of genes (Lawrence et al, 1988; Heng et al, 1992; Ried et al, 1993) and large genomic clones using the competition principles as well as chromosome painting (Lengauer et al, 1990; Lichter et al, 1990). Diagnostically, FISH allows identification of pathological chromosome abnormalities in oncology (Cremer et al, 1988; Lichter et al, 1988; Ried et al, 1992a) congenital malformation (Callen et al, 1995) and single gene disorder (Ried et al 1990). This is attributable to recent technical improvements with respect to sensitivity, multiplicity, and resolution.

In the late 1980s, non-radioactive methods of detecting genes and chromosomes were developed, resulting in techniques which make chromosomes look colorful (Langer et al, 1981). The most widely used and popular technique is “Fluorescence *In situ* Hybridisation” (FISH). In principle the FISH technique is based on molecular biology. In brief, complementary DNA complex present in chromosomes or interphase nuclei is denatured into single strands. Specific sequences of DNA (or probes) representing genes or chromosomal regions are tagged with one or more hapten molecules or fluorochrome conjugated nucleotides. These are then mixed with the single strand target DNA under appropriate conditions, for complementary binding (hybridisation) to take place. The hybridised labelled DNA sequences are visualised as fluorescent signals on chromosomes or intranuclear locations (Figure 1.8).

Figure 1.8: Strategies of FISH technique



The technique can be easily applied to both dividing and non-dividing fixed cells (interphase cells), as well as tissue sections (Klinger et al, 1992; Lawrence et al, 1990). FISH has become one of the most widely adopted techniques, because of its sensitivity, cost-effectiveness and rapid reporting turnaround time of 24-48 hours. However, it is mostly used as an adjunct to routine high resolution cytogenetics methods. The application of FISH in detecting mutations or abnormalities depends upon the availability of probes, for a particular chromosome locus. A variety of FISH probes are currently made available commercially, which are extensively used in detecting numerical and structural chromosomal abnormalities (deletion, duplications) more accurately than routine cytogenetic method. DNA probes as small as 1 kb can be visualised on metaphase chromosome (Lawrence et al, 1990; Reid et al, 1993) and interphase chromatin (Lawrence et al, 1988). Protocols for Alu-polymerase chain reaction (ALU-PCR) of yeast artificial chromosome (YAC) clones provide a better tool of interphase diagnosis, with a detection efficiency up to 99% (Lenguer et al, 1992 a, b; Lenguer et al, 1993). The probes are available in four different categories based on their property of binding, {a} centromeric probes (α -sat), {b} whole chromosome paints (WCP), {c} unique DNA-sequences, and {d} telomeric probes.

Chromosome-specific centromeric probes

These probes hybridise to repeated alphoid DNA sequences located at the centromeric/peri-centromeric region of specific human chromosomes (Moyzis et al, 1988). The centromeric probes are routinely utilised in detecting ploidy, detection of aneuploidy for specific chromosomes (21, 18, 13 or X and Y), in metaphase chromosomes, interphase nuclei (uncultured fixed cells) specimens or tumours, gametes as well as in *in vitro* fertilisation (Ried et al, 1992c). X and Y probes are widely used in the determination of sex in fetuses or in case of ambiguous genitalia (Guyot et al, 1988). Centromeric probes have also been applied in identifying marker chromosomes (Callen et al, 1990). However, centromeric probes are not suitable to detect structural unbalanced chromosomes rearrangements, as the FISH signals observed cannot be differentiated between

normal and abnormal chromosomes. Furthermore, the introduction of the primed *in situ* labelling (PRINS) technique (Koch et al, 1991) provides another way for direct detection of repeated sequences in chromosome (Gosden et al, 1991; Gosden and Lawson, 1995). It is based on the annealing of specific oligonucleotide primers and subsequent primer extension by *Taq* DNA polymerase in the presence of labelled nucleotide.

Whole chromosome paint probes

These are either biotin or digoxigenin-labelled DNA segments complementary to unique DNA segments covering the length of the target chromosome. Hybridisation of these probes produces complete fluorescent signals (appearing as paint) over the whole chromosome. Whole chromosome paints (WCP) are useful in detecting complete or partial duplication of a specific chromosome, resulting from numerical or structural rearrangements (translocation or deletion), as well as to resolve unidentified additional chromosomal segments or marker chromosomes (Cremer et al, 1988; Pinkel et al, 1988). However, chromosome paints can not be used in interphase nuclei or gametes. WCP are not useful in detecting structural rearrangements other than translocations and deletions. Whole chromosome paints are available for short or long arms of specific chromosomes, known as chromosome arm paints (CAPS) (Guan et al, 1996).

Unique DNA sequence probes (single copy probes)

These are composed of specific DNA sequences, which can be targeted against a specific 'locus' on a chromosome (chromosome band) in metaphase spreads, or interphase nuclei. The probe has its application in the precise diagnosis of submicroscopic deletions (microdeletions) or duplications not detectable by routine high resolution banding techniques. The single copy probe also is useful in detecting translocations, oncogenes, and amplified genes in malignancies in particular tumour suppressor genes (Albertson et al, 1989; Cherif et al, 1989). Unique copy probe are premixed or tagged with control probe (chromosome specific) as positive controls.

Telomeric probes

Telomeric probes are more recent, helpful in identifying subtle, terminal deletions and tandem duplications. They are composed of unique DNA sequences which hybridize to complementary sequences present at, or near the 'telomeres' [tandem repeats (TTAGGG)_n] (Moyzis et al, 1988). They are specific for either short arm (p) or long arm (q), of a chromosome. The telomeres are mostly G-band negative.

Multiplex FISH

Recent advances in FISH technology supported by sophisticated computer software (Smart Capture), filters sets and digital mapping devices such as charge coupled device (CCD) cameras increased the sensitivity of FISH. Multiple haptenisation and detection protocols, and combinatorial labelling approaches allow the simultaneous visualisation of several target regions in metaphase chromosomes and interphase nuclei (Nederlof et al, 1990; Ried et al, 1992b; Dauwerse et al, 1992; Wiegant J, 1993; Tocharoentanaphol et al, 1994).

Finally, the application of FISH technology in routine cytogenetic analysis has helped in the precise diagnosis of several cases of mosaicism, structural rearrangements, in particular, microdeletions and marker chromosomes, particularly the X and Y chromosomes (Callen et al, 1995; Hatchwell et al, 1996; Bettio et al, 1997; Henegariu et al, 1997). FISH has tremendous applications in cancer cytogenetics. However, FISH does not replace banding, but it aids in the detection of aberrations precisely.

(C) Southern blot hybridisation:

Another technique with wide application is Southern blot method. Essentially, the test DNA is digested with one or more restriction enzymes, size-fragmented by agarose gel electrophoresis, denatured, and transferred to a membrane for hybridisation (Southern et al, 1975). The main advantage of the technique is that no detailed knowledge of the sequence and the structure of a gene is required and a preliminary screen can be done with a probe of interest immediately after its isolation (Grompe M, 1993). Large deletions, duplications, and insertions can be detected by this method. In X-linked disorders such as

Duchenne muscular dystrophy (DMD), deletions result in complete absence of a sequence in an affected male. This is readily detected on a southern blot with a cDNA probe, because a DNA fragment will be absent or have a different length (Koenig et al, 1987). For autosomal genes and for female carrier of X-linked disorders deletion detection is more difficult. Quantitative Southern analysis can be done, but such approach can be laborious and subject to error (Abbs et al, 1991). Occasionally, the deletion results in junction fragment which can be helpful in heterozygotes detection. In some, a mutation creates or destroys a restriction enzyme recognition site which may be detected by southern analysis. Finally, Southern analysis has been used successfully to detect large trinucleotide repeat expansion in patients with FRAXA and myotonic dystrophy (Fu et al, 1991; Harley et al, 1992a).

(D) PCR-based detection methods:

Since the advent of PCR technology using a thermostable DNA polymerase, which allow the in vitro production of large amount of a target DNA sequence, various PCR-based methodologies have been developed for rapid detection of genetic variation (Grompe M, 1993; Cotton R, 1993). Both, genomic DNA and mRNA can be used as templates for the PCR-based mutation strategies. The advantage of using mRNA which is reverse transcribed to cDNA as template, is that only the coding region will be analysed, leading to the rapid detection of gross deletions or alterations, minor alterations, or splicing errors.

Multiplex PCR for the detection of deletion:

Because 60% of dystrophin gene mutations are deletions, a rapid and efficient method of screening for deletions was introduced by Chamberlain et al (1989) to analyse six frequently deleted exons simultaneously in a multiplex amplification. The multiplex reaction efficiently detected 80-98% of all dystrophin gene deletions (Chamberlain et al, 1989; Abbs et al, 1991). Recently, Tuffery et al (1998) developed a multiplex PCR to analyse 17 exons of the dystrophin gene in one reaction. Therefore, this method has found wide use in the diagnosis of DMD disorder. The deletions in homozygotes are indicated by the absence of some of the bands in the multiplex pattern, while in heterozygote, the deletions are seen as 50% reduction of band intensities in a quantitative analysis of the multiplex

reaction (Abbs and Bobrow, 1992). Since then the multiplex PCR applied to loci of known sequences prone to deletions and had been employed with other genes such as the 8-fragment multiplex PCR reaction of the human hypoxanthine guanine phosphoribosyl transferase (*HPRT*) gene (Gibbs et al, 1990; Grompe M, 1993).

1.7.1.2 Detection of single base changes and small sequence alterations

Single base alterations are the most common type of mutation of most loci. Changes of a single base can result in the insertion of incorrect amino acid or can change an amino acid to a stop codon. Some changes are conservative, substituting an amino acid with similar properties that may not affect the function of the protein, and represent polymorphisms and common genetic variants. In other instances, non-conservative changes result in an alteration of protein function or changes of an amino acid codon to a stop codon, causing premature termination of protein synthesis. Point mutation can also occur within an intron, where they may affect the fidelity of splicing (Krawczak et al, 1992).

The detection of point mutation and small sequence alterations in a DNA sequence represent technical obstacles for the genetic analysis of human inherited disease. To face this problem many methods have been developed, particularly after the introduction of PCR (Grompe M, 1993; Cotton R, 1993). Mutations can be detected by: (1) analysis of single strand conformational polymorphisms (SSCP; Orita et al, 1989), (2) heteroduplex analysis (HA; White et al, 1992), (3) denaturing gradient gel electrophoresis (DGGE; Fischer and Lerman, 1983; Myers et al 1987) (4) chemical cleavage mismatch (CCM; Cotton et al, 1988) or RNase cleavage (Myers et al, 1985a).

(A) Single strand conformation polymorphism (SSCP) analysis:

Owing to its simplicity and sensitivity SSCP is one of the most widely used method for the detection of point mutation. It is based on the fact that single-stranded DNA has a tendency to fold up and form complex structures stabilised by weak intramolecular bonds, and the electrophoretic mobility of such structure on non-denaturing gel will depend not only on their chain length but also on their

conformation, which in turn depends on its nucleotide sequence (Orita et al, 1989). With this method, PCR products are partially denatured to allow the formation of single stranded secondary structures and then electrophoresed in a non-denaturing polyacrylamide gel. A test DNA sample that differs by a single base from a standard DNA sample can be detected as a comparative mobility shift during electrophoresis if the base results in a change of conformation. There are however, discrepancies in the reported sensitivity of this technique (Hayashi K, 1992; Sarkar et al, 1992 a, b; Hayahi and Yandell, 1993). Detection rates reported varies from 79% to 96% when PCR product of 200 bp or less and rapidly falls to <50% when the size is more than 400 bp (Hayashi K, 1991; Grompe M, 1993; Sarkar et al, 1992 a, b). Several technical factors that might affect sensitivity, including the type of gel matrix, electrophoresis condition, the dimension of the gel, overloading the gel, and the isotope used (Dianzani et al, 1993). The sensitivity of SSCP is improved when the analysis is carried out on an RNA copy of the sequence, since the secondary structure of RNA is more suited to SSCP (Dianzani et al, 1993).

(B) Heteroduplex analysis:

Heteroduplex analysis (HA) takes advantages of the formation of heteroduplexes between mutant and wild type sequences. Heteroduplex molecules containing single base pair mismatches can be accurately separated from related nonhomoduplex on nondenaturing gel (Keen et al, 1991; White et al, 1992). This phenomenon is thought to be caused by sequence dependent conformation changes in the double strand DNA induced by mismatch. Although HA is as simple as SSCP, its application has not been so widespread, because of its later description and because of the need for Hydrolink gels in the initial description (Keen et al, 1991). Recently, new gel matrices (Hydrolink and MDE from AT Biochem) (Grompe M, 1993) have become available, which markedly enhances the ability to detect mutation induced mobility shifts in heteroduplex molecules. Heteroduplex analysis on Hydrolink gel has been successfully applied to mutation screening in several genetic diseases. This simple technique was reported to be able to identify different types of genetic alteration including point mutation and minor deletion or insertion in PCR fragments of different sizes from about 200 up to 1400 bp (Tassabehji et al, 1992). The main disadvantage of HA is the lack of 100% detection as in SSCP analysis (80-90%) and the sensitivity increase with

decreasing the size of the DNA fragment (optimal <300 bp) (Perry et al, 1992; White et al, 1992). Both isotopic and non-isotopic methods have been used (Perry et al, 1992; White et al, 1992; Yap and McGee, 1992).

(C) Denaturing Gradient Gel Electrophoresis (DGGE):

This method of mutation detection is developed by Myers et al (1985a, b), compares homoduplexes with heteroduplexes of wild type and mutant DNA strands in terms of their electrophoretic mobility. In principle, when a double stranded DNA (dsDNA) fragment is subjected to electrophoresis through a gradient increasing concentration of a denaturing agent it will reach a certain point in the gradient where its lowest melting temperature domain melt to create a branch molecule which effectively does not move any further through the gel matrix. The melting behaviour is sequence dependent. Strand separation at a domain in a DNA fragment is detected as a reduction in the mobility of the fragments. Two dsDNA fragments of the same sizes but differing in sequences, melting at different points in a DGGE and can be distinguished by differential migration.

A major draw back of DGGE technique initially was its insensitivity to mutations in regions of the DNA which melt last in the gradient of denaturant, because at this point the strands separate and the resolution is lost. To observe the effect of mismatches in the highest melting domain, a modification was designed that involved the addition of a GC-rich fragment (Myers et al, 1985b; Myers et al, 1985c). Detection is usually carried out by a non-radioactive means (Fodde et al, 1994). DGGE can detect mutations with 95% accuracy in PCR products of up to 600 bp in length (Grompe M, 1993).

(D) Chemical Cleavage Mismatch Detection:

The cleavage methods of mutation detection are also based on the principle of detecting differences between wild type and mutant single-stranded molecules when a heteroduplex is formed between the two. In RNase cleavage (Myers et al, 1985a) a ribonucleic acid probe hybridised to RNA or DNA containing one base difference can be cut at the mismatch by ribonuclease (RNase A). Mutation can then be detected and located after analysis of the cleavage products by gel electrophoresis (Dianzani et al, 1993). However, the method is not very sensitive,

it detects only one third to two third of point mutations (Dianzani et al, 1993; Myers et al, 1985a). Given the limitation of the enzymatic cleavage methods of mutation detection, Cotton et al (1988) developed an assay for detection of heteroduplex mismatches using Maxam and Gilbert cleavage chemistry (Maxam and Gilbert, 1980). The mutation screening by chemical cleavage mismatch method (CCM) is based on the fact that mismatched cytosine (C) and thymidine (T) are much more reactive with the compounds, hydroxylamine and osmium tetroxide, respectively, than are the matched pair C and T. Double strand DNA treated in this way can then be cleaved opposite the modified bases by piperidine and the fragments separated by gel electrophoresis. The main advantages of CCM are high detection rate (79-100%; Forrest et al 1991) and the ability to screen kilobase lengths of DNA or RNA (up to 1.7 kb; Grompe M, 1993). Moreover, CCM gives in the approximate location of the mutation by estimating the size of the cleavage product as well as the type of base change from the cleaving reagent.

While all of these methods have been used successfully for identification of alleles, which cause disease, SSCP (because of simplicity) and DGGE (because of its near 100% sensitivity) are the favoured techniques. A draw back of SSCP is that two or three different gel conditions are required to detect all mutation in fragments and DGGE suffers from the need for special equipment and more expensive GC-clamps (Dean M, 1995). Moreover, some methods are not suitable for screening large numbers of individuals, because of their complexity. This is the case for RNase cleavage and CCM methods.

(E) Direct sequencing:

The ultimate molecular characterisation of a mutation is at the level of the nucleotide sequence and with the availability of the PCR technique, sequence information has become readily available. Sequencing can be used either as a screening or a diagnostic method. Direct sequencing of PCR products has been used as the primary mutation screening method to detect mutation in parts of large genes (Higuchi et al, 1990) or by sequencing an entire coding sequences (Mogne et al, 1992).

The methods of Sanger (1977), Maxam and Gilbert (1980) for rapid DNA sequence determination have formed the corner stone of the sequencing technique used today. Of the two methods, Sanger's one is most widely used. The Maxam

Gilbert technique for sequencing DNA is based on a chemical procedure that breaks a terminally labelled DNA molecule partially at each repetition of a base. The lengths of the labelled fragments then identify the position of that base. They described a reaction that cleaves DNA preferentially at guanine, adenines and guanines equally, at cytosine and thymines equally, and at cytosine alone. When the products of these four reactions are separated by high resolution denaturing polyacrylamide gel electrophoresis, the DNA sequence can be read from the pattern of radioactive bands.

The Sanger's method also known as dideoxy sequencing or chain termination is an enzyme-based method. In this approach the usual first step is to prepare a single-stranded DNA from the DNA to be sequenced. A chemically synthesised oligonucleotide primer is annealed to its complementary sequence on a single stranded DNA template. The primer/template duplex is then used as a substrate of chain extension from the 3' end of the primer by DNA polymerase. Four separate synthesis reactions are carried out. Each include all four nucleotide dATP, dCTP, dGTP, and dTTP, one of which carries a radioactive isotope, together with one of the four dideoxy nucleotide (ddNTP) analogue, ddATP, ddCTP, ddGTP, and ddTTP. Incorporation of ddNTPs causes chain termination since they lack a 3'-hydroxyl group. Random low level incorporation of a specific ddNTP, in competition with the normal dNTP analogue, will result in mixture of different length chains, all starting at the 5' end of the primer and ending at every possible position where the specific ddNTP can be incorporated in place of the dNTP. The average length of the resulting chain can be altered by changing the ddNTP/dNTP ratio within the reaction mixture. For example increasing relative concentration of ddNTP will result in a short average chain length. These four sets of reactions are then fractionated alongside each other by denaturing polyacrylamide gel electrophoresis. On conventional sequencing gels about 200-400 nucleotides can be determined from a single DNA sample.

1.7.2 Methods to detect known sequences alteration (diagnostic techniques)

Once a particular disease causing mutation has been identified in a family, diagnostic techniques are called into use. The genetic screening programmes of the general population are aimed at preventing common genetic diseases. There are

several approaches for conducting genetic screening which dependent on the target population and the type of the disease (Caskey T, 1993). Genetic screening is offered to populations at high risk of autosomal recessive disorders such as Tay-Sach disease (Kaback M, 1981), hemoglobinopathesis (sickle cell anemia or thalassemia) and lately for cystic fibrosis (Scriver and Fuiwara, 1992). These techniques are generally simpler than those used in the original identification of mutation. This can be achieved by the following methods; (1) allele specific oligonucleotide hybridisation (ASO) (Wallace et al, 1979), (2) allele specific PCR amplification or by an amplification refractory system (ASA/ARMS) (Wu et al, 1989; Newton et al, 1989a), (3) restriction enzyme digestion (if a site has been created or abolished), (4) artificial introduction of a restriction site (AIRS)(Cohen and levinson, 1988), or (5) SSCP or HA. Direct sequencing of PCR products may also be the method of choice.

(1) Allele-specific oligonucleotide (ASO) hybridisation

A point mutation, which does not produce a restriction site change, may still be detected using allele-specific oligonucleotide (ASO) probes. Oligonucleotide probes can therefore be designed to hybridise to specific alleles of a gene, which differ by a single nucleotide at a diagnostic site. Two probes are made, one has the normal sequence, while the other is identical except for a single altered base corresponding to a particular mutation (Wallace et al, 1979). ASO probes are typically 15-20 nucleotides long and are normally employed under stringent hybridisation conditions at which the DNA duplex between probe and target is only stable if there is perfect base complementarity between them. ASOs can be used in conventional Southern blot hybridisation, it is more convenient to use them in dot-blot assays. Both radiolabelled and fluorescently labelled probes have been used. In reverse blot (Saiki et al 1989) different probes were spotted onto the membrane and the biotin-labelled target sample applied. The probes forming duplexes without mismatches and retaining the target became stained with HRP avidin. The approach is feasible if the DNA sequence causing a genetic disease is known and if the number of disease-causing mutations is limited (such as sickle cell disease and cystic fibrosis). DNA from normal individuals will hybridise only to the wild-type oligonucleotides, heterozygotes will hybridise to both, and individual homozygote for the mutation will hybridise to only to the

mutant oligonucleotide. Multiple mutations can be detected in a single reaction by mixing several hybridisation probes (Saiki *et al.* 1986).

(2) Amplification refractory mutation system (ARMS)

The PCR depends on elongation from the 3' end, by *Taq* polymerase, of a double-stranded template to the target DNA. Naturally, if the template is mismatched at the 3' end, the PCR will proceed less efficiently and, over 20 or 30 cycles, this will have a significant effect. In the amplification refractory mutation system (ARMS) two sets of primers are used, each set designed in such a way that amplifies only either the normal or the mutant allele. Primers that are mismatched with either target sequence by only single nucleotide at their 3' ends will not amplify the DNA to which they anneal. Two parallel reactions are run; one will prime with oligonucleotides corresponding to the wild type sequence, and a second corresponding to a known mutation. The former will amplify normal copy of the gene, while the later will amplify the mutant copy (Newton *et al.*, 1989a). DNA from heterozygote individual will amplify with both primers. A second pair of primers, producing a different size of product, is normally introduced in the same reaction as a positive control. ARMS have found wide spread use in the diagnostic laboratory and are routinely used for diagnosis of thalassemia (Old *et al.*, 1990) and cystic fibrosis (Newton *et al.*, 1989b).

(3) Artificial introduction restriction sites (AIRS)

Many mutations, but not all, naturally create or remove restriction enzyme sites. The artificial introduction of restriction site system (AIRS) provides an alternative way of making restriction enzyme detection of sequence changes, which is more universally applicable (Cohen and Levinson, 1988). A mismatch is deliberately introduced, by a primer that has one base mismatched near to the site of mutation, to create an artificial restriction enzyme site in the PCR product, either in the normal or mutant cases. Therefore, presence of the mutation will make the proper recognition site for a specific restriction endonuclease. Digestion of the PCR products using this enzyme followed by gel electrophoresis will enable the identification of the presence or absence of the mutation in the sample. AIRS have been used successfully to screen for common mutations in different genes such as Duchenne muscular dystrophy (Yau *et al.*, 1993) and phenylketonuria (Eiken *et al.*, 1991).

1.8 Aims of the present project

The overall aims of the present project are to optimise and apply methods that detect large or small sequence DNA alterations in patients with sex chromosome aberrations and sex determination / differentiation abnormalities. These patients were chosen because of the differences with regard to known molecular pathology, available gene information and the availability of studying material (DNA or chromosomal fixed materials). The spectrum of the mutations ranges from cytogenetically visible chromosome abnormalities to microdeletions and finally to single base alterations. This will involve the application of cytogenetic, molecular cytogenetic and molecular genetic investigations. The specific targets are summarised as follows:

I) Cytogenetic and molecular cytogenetic studies

- i) To optimise the FISH technique using different probes such as the repeated sequences (e.g. the alpha satellite centromeric probes of X and Y chromosomes), whole chromosome libraries specific for both the X and Y chromosomes (chromosome painting), and X and Y-specific probes.
- b) To optimise multi-colour FISH using the optimised probes in the uni-colour FISH experiments.
- iii) To apply FISH to diagnostic problems in clinical cytogenetics in patients with sex chromosome abnormalities.
- iv) To analyse the X-inactivation pattern using BrdU labelling (late replication) methods of structurally abnormal X chromosomes particularly ring X chromosomes and to correlate it with the phenotype if it is possible.

II) Molecular Genetic studies:

- i) To detect and fully characterise mutations in the androgen receptor gene (*AR*) in patients with androgen insensitivity syndrome (AIS).
- ii) To detect mutations in the sex determining (*SRY*) gene in patients with sex reversal (46,XY female).

- iii) To detect Y sequences, particularly of the *SRY* gene in males with female karyotypes (46,XX male).
- iv) To develop diagnostic screening methods for mutation detection in male patients with non-obstructive infertility. The screening programme is based on:
 - (1) microdeletion detection in azoospermia factor (AZF) region(s) by optimising and applying multiplex PCR using 28 different pairs of primer sets.
 - 2) To detect microdeletions of the *RBM* gene family by applying FISH and Southern blotting techniques using MK5 and Mk29 cDNA probes (specific for *RBM* gene family) as a first step of the screening method.
 - 3) To carry out a comprehensive search for mutations in the *RBM1* gene by designing primers spanning the entire essential sequence of the gene to enable PCR-based screening such as SSCP, HA, and sequencing.

CHAPTER 2

MATERIALS AND METHODS

(2) MATERIALS AND METHODS

2.1 Patients

A total of 93 patients with known or with possible sex chromosome abnormalities were investigated. Table 2.1 summarises the patient categories and the techniques applied for studying each category.

Twenty four patients were referred from Duncan Guthrie Institute (DGI) and from Kuwait Medical Genetic Centre with known or suspected X and Y chromosomes abnormalities. These patients were studied at cytogenetic and molecular cytogenetic level. Out of these seven patients with Turner phenotype carrying ring chromosomes and one patient with 46,XXp+ karyotype were studied for late X replication.

Thirteen of the investigated patients were referred due to sex disorders. Four male patients with 46,XX karyotypes were investigated by cytogenetics, molecular cytogenetics and by molecular genetics for the presence or absence of Y chromosome material. Five females with 46,XY karyotype were clinically suspected to have androgen insensitivity syndrome, while 4 females with 46,XY karyotype were clinically classified as gonadal dysgenesis syndrome.

Blood samples were collected from two different male infertility clinics in Kuwait from a total of 56 males with azoospermia (sperm number of zero) or severe oligospermia (sperm count < 2 million/ml).

2.2 materials and Solutions:

Supplemented RPMI 1640

To 100 ml of RPMI 1640 the following were added:

| <u>Material</u> | <u>Final concentration</u> |
|--|-----------------------------------|
| PHA | 2% |
| Fetal calf serum | 20% |
| L-Glutamin | 2% |
| Penicillin/Streptomycin (5000 Unit/ml/5000 µg/ml) | 1% |

Stored at 4°C.

1x Iscoves Medium

To 100 ml of 1 x Iscoves media the following were added:

| <u>Material</u> | <u>Final concentration</u> |
|-------------------------|-----------------------------------|
| Heparin (2000 Unit/ml) | 1% |
| L-glutamin | 1% |
| PHA | 2% |
| Penicillin/Streptomycin | 1% |

The media was stored at 4°C.

Hypotonic Solution (0.075 M potassium chloride)

For every 100 ml deionized water 0.56 gm of KCl added and stored at room temperature for up to two weeks.

Carnoy's Fixative

3 parts methanol to 1 part glacial acetic acid prepared fresh each harvest.

Normal Saline

0.09% NaCl (sodium chloride)

Sorensens Buffer pH 6.8

(a) 10 X anhydrous potassium phosphate monobasic (KH_2PO_4) M/15

9.09 gm KH_2PO_4 dissolved in 100 ml distilled H_2O . Stored at 4°C.

(b) 10 X Anhydrous Sodium phosphate dibasic M/15

11.88 gm Na_2HPO_4 dissolved in 100 ml distilled H_2O . Stored at 4°C.

Working buffer was prepared by mixing 5 ml of K_2HPO_4 and 5 ml of NaH_2PO_4 in 90 ml distilled water. Adjusted PH to 6.8 and stored at room temperature.

10% stock trypsin solution

200 mg trypsin 1:250 (Difco) reconstituted with 20 ml sterile normal saline.

Aliquoted in 1.0 ml lots and frozen.

0.02% working trypsin solution

1 ml 10% stock trypsin diluted in 49 ml normal saline. Prepared fresh each harvest.

Giemsa Staining solution

Two gm of Giemsa powder dissolved in 132 ml of glycerol. Incubated in water bath for 2 hours at 56°C, cooled and then 132 ml of methanol was added. The mixture was then shaken well and kept in a dark place for seven days. The stain was then filtered through Whatman 1MM paper and stored in brown bottles for use.

2% Giemsa solution

1 ml Giemsa added to 50 ml (PH 6.8) working Sorensens buffer solution.

Leishman staining

3 gm of Leishmans powder dissolved in 2000 ml of methanol, filtered through Whatman 1 MM paper and used

Fluorodeoxy uridine (FUdR) stock solution (4×10^{-4} M)

To 50 ml distilled water added 5 mg FUdR stored frozen in dark vials.

Uridine stock solution (3×10^{-3} M)

To 10 ml distilled was added 10 mg uridine and stored frozen as small aliquots.

BrdU solution (1.5 mg/ml: working solution)

To 17 ml distilled water added 30 mg BrdU, 2 ml uridine stock solution, 1 ml FUdR stock solution. Filter sterilised and stored frozen in dark vials. This solution can be used over a period of 2 weeks.

Hoechst 33258 solution (150 μ g/ml)

To 100 ml distilled water added 15 mg Hoechst 33258. Stored frozen in dark vials.

Phosphate Buffer PH 6.8

Buffer consisted of 0.025 M KH_2PO_4 (3.4 gm/L) titrated to PH 6.8 with 50% NaOH (sodium hydroxide).

2X SSC solution

To 1000 ml distilled water added 17.5 gm NaCl, 8.8 gm sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$).

Staining Solution

1 part Leishmans stain, 3 parts phosphate buffer

Luria-Broth Media (LB)

To 450 ml of deionised water the following were added:

| | |
|---------------------|--------|
| Bacto-tryptone | 5.0 gm |
| Bacto-yeast extract | 2.5 gm |
| NaCl | 5.0 gm |

Adjusted PH to 7.0 with concentrated NaOH. The volume was adjusted to 500 ml with deionised water and then sterilised by autoclaving at 121°C for 20 minutes. The medium was cooled and then an appropriate antibiotic with final concentration of 60-100 µg/ml was added under sterile conditions and kept at 4°C until used.

SOC Media (1.020 litre)

10 gm bactotrypton
5 gm bacto yeast extract
5 gm NaCl

PH 7.5, adjusted by 5M NaOH.

Sodium citrate (1M)

29.4 gm of sodium citrate was dissolved in 90 ml of ddH₂O. The PH was adjusted to 5.8 and then the volume was adjusted to 100 ml with dH₂O.

EDTA (0.1 M)

3.2 gm of EDTA was dissolved in 90 ml of dH₂O. The pH was adjusted to 8 and then the volume was adjusted to 100 ml with dH₂O.

5M Potassium Acetate

4.9 gm of potassium acetate was dissolved in 10 ml H₂O.

T.E. buffer

10 mM Tris-HCl was mixed with 1 mM EDTA, PH 8.6. The obtained solution was filter sterilised and stored at room temperature.

10 x T.B.E. buffer

The following were added:

| | |
|------------|--------|
| Tris base | 108 gm |
| Boric acid | 55 gm |
| EDTA | 9.3 gm |

To 800 ml of dH₂O and dissolved. The pH was adjusted to 8.1-8.2 with 1 N NaOH. The volume was adjusted to 1 litre by dH₂O and stored at room temperature.

T.A.E. buffer 50X

| | |
|---------------------|---------------------------------|
| Tris base | 242 gm |
| Glacial acetic acid | 57.1 ml |
| 0.5M EDTA, PH 8.0 | 100 ml |
| | dH ₂ O up to 1 litre |

6 X Agarose gel loading Mix

| | |
|----------------------------|-----------|
| Bromophenol blue | 0.25 gm |
| Sucrose | 40% (w/v) |
| Dissolved in 1X TBE buffer | |

Salmon Sperm DNA (20 mg/ml)

200 mg salmon sperm DNA dissolved in 10 ml of distilled water and incubated in a 70°C water bath for 10 minutes. The solution then sheered by repeatedly passing through a needle (21G) attached to a 10 ml syringe. The tube then sonicated for 3x4 minutes at 4 volts. The fragment size of sonicated DNA was checked by gel electrophoresis in 0.8% agarose gel and stored at 4°C.

100X Denhardt's solution

| | |
|----------------------------|----------|
| BSA (bovine serum albumin) | 2% (w/v) |
| Ficoll | 2% (w/v) |
| Plyvinylpyrrolidone (PVP) | 2% (w/v) |

Hyrbridisation buffer 1

| | |
|-------------------------------|-----------|
| 5 ml Formamide (Fluka) | 50% (v/v) |
| 2 ml 50% dextran sulphate | 10% (w/v) |
| 0.5 ml 20 X SSC | 1 X SSC |
| 2.5 ml sterile water | |
| Aliquoted and stored at -20°C | |

Hybridisation buffer 2

Two gm of dextran sulphate was dissolved in 4 ml of deionised water and then the following were added:

| | |
|-----------------------------|---------|
| Formamide (Fluka) | 10 ml |
| 20 x SSC | 2 .0 ml |
| Salmon Sperm DNA (10 mg/ml) | 200 µl |

The contents were mixed very well, aliquoted in 1ml volumes in eppendorf tubes and stored at -20°C until used.

20X SSC

Tri-sodium citrate 44.1 g

Sodium chloride 87.6 g

Dissolved in dH₂O, adjusting the volume to 500 ml and stored at room temperature.

4X SSC, Tween-20

100 ml of 20 X SSC diluted in 400 ml dH₂O then 250 µl of tween-20 was added.

The solution was mixed and stored at room temperature.

Wash A solution

20 X SSC 100 ml

Tween-20 250 µl

Dried skimmed milk 25 gm

The volume was adjusted to 500 ml with dH₂O, mixed well and then spun at 200 rpm for 15 minutes. The solution was freshly prepared for every experiment.

DAPI (4,6-diamidino-2-phenyl-indole)- working solution (40 µg/ml)

1 µl of DAPI (mg/ml) was diluted in 24 µl of distilled water, mixed and stored in dark at 4 °C until used.

Propidium iodide - working solution (20 µg/ ml)

2 µl of propidium iodide (10 mg/ml) was diluted in 988 µl of distilled water, mixed and stored in dark at 4 °C.

T_{0.1}E buffer

10mM Tris-HCl, PH 7.4

0.1mM EDTA

20X TTE

216 g Tris base

72 g taurine

4 g EDTA

distilled water up to 1 litre

CIRCLEPREP Kit BIO 101: Cat. No. CP-100)

The kit contains the following:

- Pre-lysis buffer (Tris/EDTA/ glucose solution)
- Alkaline lysis reagent (0.2 N NaOH and 1% SDS).
- Neutralising solution (3M Potassium acetate)
- Lithium Chloride solution (near saturated solution)
- Circleprep Glass milk
- Binding buffer (KBr/NaI/Tris mixture)
- Wash solution
- Sieve material

BRL Nick Translation Kit (GIBCO BRL, Cat. No. 8160SB)

The kit supplied the following reagents and solutions:

- Solution A4: 0.2mM nucleotides C, G and A in 500 mM Tris-HCL (PH 7.8), 50 mM Magnesium Chloride.
- Solution C: 0.4 units/ μ l DNA polymerase I, 40 pg/ μ l DNA Pol I/DNase I, 50 mM Tris-HCl, pH 7.5, 5 mM Magnesium acetate, mM 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 50% glycerol and 100 μ g/ μ l bovine serum albumin).
- Solution D: 300mM disodium EDTA, pH 8.
- Solution E: dH₂O.

Digoxigenin DNA labelling Kit (Boeshringer Mannheim Biochemica' Cat.Bo 1175033)

The kit contains the following:

- Unlabelled control-DNA 1: One vial with 20 µl pBR328 DNA, 100 µg/ml.
- Unlabelled control-DNA 2: One vial with 20 µl pBR328, 200 µg/ml, linearised with BamH 1.
- DNA dilution buffer: Two vials with 1 ml of 50 µg/ml herring sperm DNA in 10 nmol/l Tris-HCl and 1 mmol/l EDTA, pH 8.0 each.
- Labelled control DNA: One vial with 50 µl linearised pBR328 DNA, labelled with digoxigenin.
- Hexanucleotide Mixture: One vial with 80 µl 10 X concentrated hexanucleotide reaction mixture.
- dNTP Labelling Mixture: One vial with 80 µl of 10 X concentrated dNTP labeling mixture containing dATP, 1 mmol/l ; dGTP, 1 mMol/l ; dTTP , 0.65 mmol/l; Dig-dIT[, 0.35 mMol/l; pH 7.5.
- Klenow Enzyme, labeling grades: One vial with 40 µl Klenow enzyme labelling grade, 2 Units/µl.

Slide cleaning

Slides are cleaned in absolute alcohol before use to facilitate uniformity of spreading and to increase metaphase quality and banding quality. Slides are soaked briefly in ethanol, wiped with a tissue or gauze, and then dipped back into the absolute alcohol before dipping in distilled water. Alternatively slides are soaked in detergent (100% Decon 90: Decon Laboratories Limited) then slides were rinsed thoroughly under running tap water in order to clean them completely from the detergent. Slides then stored in 70% alcohol till use. On the day of using the slides, slides were rinsed with tap water completely to get rid of alcohol.

Table 2.1: Summary of cases investigated and techniques applied.

| Subjects | No. of Cases | Remarks/Ref. Diagnosis | Techniques Applied |
|---|--------------|--|----------------------------------|
| A] Known or suspected chromosomal abnormalities | 24* | (a) Turner phenotype = 16 (b) Primary amenorrhea = 2 (c) Failure to thrive; developmental delay = 2 (d) MCA = 2 (e) Ambiguous genitalia = 1 (f) Short stature = 1 | Cytogenetic FISH |
| B] Sex-reversal | 13 | | |
| i) 46,XX males = 3 | | Infertility | Cytogenetic FISH |
| ii) 46,XX male = 1 | | Hypogonadism; Undescended testes | Molecular |
| iii) 46,XY females = 5 | | CAIS = 3 PAIS = 2 | Cytogenetic Molecular |
| iv) XY gonadal dysgenesis = 4 | | Pure gonadal dysgenesis = 2 Incomplete gonadal dysgenesis = 2 | Cytogenetic FISH Molecular |
| C] Male infertility | 56** | a) Azoospermia = 32 b) Severe oligospermia = 24 | Cytogenetic FISH Molecular |
| Total | 93 | | |

* Refer to Table 3.1 for detailed description of chromosomal abnormalities.

** Refer to Table 3.5 for detailed description.

CAIS = Complete androgen insensitivity syndrome; PAIS = Partial androgen insensitivity syndrome. MCA = Multiple congenital anomalies

2.3 Cytogenetic studies

2.3.1 Chromosomal preparation from stimulated peripheral blood

In principle, blood is cultured for 72 hours in media containing mitogen [usually phytohemagglutinin (PHA)] which is added to stimulate lymphoproliferation. Before harvesting the culture is treated with colcemid to arrest the cell cycle in metaphase. The culture is then treated with a hypotonic solution to swell the lymphocytes and is subsequently fixed in a methanol: acetic acid solution.

10 ml of peripheral blood sample was collected in a sterile 10 ml lithium heparin tube and gently mixed to prevent clotting. 0.1 ml of blood was added to 10 ml complete RPMI 1640 supplemented media or 1 ml of blood cultured in 10 ml 1 x Iscoves medium and the cultures were incubated at 37°C for 72 hours. 0.1 ml of colcemid (10 µg/ml) was added to each 10 ml culture and incubation at 37°C was continued for an additional 30-60 minutes. The culture tubes were then centrifuged at 1200 rpm (400g) for 8 minutes using Beckman GH-3.7 rotor in GS-6 centrifuge (Beckman, California). Carefully the supernatant was removed and discarded into 1/10 volume Chlorox and the pellet were resuspended in 10 ml of 0.075 M KCl. The tubes were incubated for 15-20 minutes at 37°C and then centrifuged at 1200 rpm (400g) for 8 minutes. The pellets were resuspended in 10 ml of fresh fixative by flicking, pipetting or vortexing and the tubes left to stand at room temperature for 30-60 minutes. The tubes were then centrifuged at 1200 rpm for 8 minutes and the pellets washed in fresh fixative. This step was repeated 2-3 times before the cells were resuspended in 10 ml fixative and stored at 4°C or at -20°C until needed.

2.3.2 Slide preparation

Chromosome pellets removed from 4°C or -20°C storage and allowed to come to room temperature. The tubes were centrifuged at 1200 rpm for 8 minutes

resuspended in 2-3 ml fresh fixative until the solution was just slightly hazy. A few drops (3-5 drops) of cell suspension were dropped onto wet cold slides from approximately 15 cm distance and slides allowed to air dry. The slides were viewed under phase contrast (10x objective) to evaluate mitotic index, metaphase quality and chromosome morphology.

2.3.3 Giemsa banding

Giemsa banding (G-banding) has become the most widely used technique for the routine staining of mammalian chromosomes. The most common methods to obtain this banding pattern are to treat slide with a proteolytic enzyme such as trypsin (Seabright, M, 1971) or to incubate the slides in hot saline citrate (Sumner et al, 1971), although a variety of other methods have been used.

In this study G-banding was produced on freshly made metaphase spreads by a modification of Seabright 1971 method. In general, slides for G-banding should be aged 3-5 days at room temperature or overnight (16-18 hrs) at 56-60°C for optimal results. To get optimal result of G-banding slides were aged overnight at 56-60°C. Slides were then treated for 20-40 seconds in 0.02% working trypsin solution (10% stock solution). Slides then rinsed thoroughly with normal saline stained for 5 minutes in 2% Giemsa solution, rinsed in distilled water, and then air dried. 50 cells were counted and 5 cells were analysed for full karyotyping for all cases using Axioplan Carl-Zeis light microscope.

2.3.4 Late replication study of X-chromosome LRX [Reverse banding using 5-Bromodeoxy uridine (BrdU) and Giemsa (RBG)]

Chromosomes and their segments replicate at different times during the S phase. This phenomenon has been extensively studied both itself and for its implications for chromosomes structure and function.

This technique can be achieved for example by growing lymphocytes in thymidine-containing-medium and then a medium with BrdU. The late replicating chromosomes including inactive X chromosome are darkly fluorescent with quinacrine and faintly stain with Giemsa. Yet, the inactive X is not completed

inactive, there are several segments escaping inactivation especially the pseudo autosomal segment Xp22.3 and the postulated inactivation centre at Xq13.1 (Rooney and Czepulkowski, 1992).

Differential replication studies were conducted using 1 ml of peripheral blood in 9 ml 1x Iscoves culture medium containing PHA. Cultures were then incubated at 37°C for 63 hours. 0.2 ml BrdU solution (1.5 mg/ml) was added to each culture and incubated in the dark (using aluminium foil) for a further 6 hours at 37°C. Then cultures incubated with 0.1 ml of colcemid in dark at 37°C for a further 45 minutes. Cultures then harvested as normal as described previously taking care not to expose cultures to light in order to prevent photodegradation of BrdU. The incorporation of BrdU was detected using Hoechst/Giemsa staining procedure with some modification according to Latt (Latt SA 1973). To each prepared slide few drops of Hoechst 3358 solution (150 µg/ml) were added, covered with a cover slip (22X 50 mm) and allowed to stand for 15 minutes in the dark at room temperature. The cover slips were carefully removed by forceps taking care not to scratch the slide. Slides were then rinsed in distilled water and allowed to air dry. The slides were mounted in 2 x SSC with cover slips and exposed to ultraviolet (long wave) at a distance of 20-25 cm for 2 hours. Slides then rinsed in distilled water and air-dried. The slides then stained in freshly prepared Leishmans stain for 2 minutes, mounted in Histoclear and were viewed with a Zeiss Axioskop microscope.

2.4 Molecular cytogenetics: Fluorescence in situ hybridisation (FISH)

Fluorescence in situ hybridisation (FISH) involves hybridisation of biotin or digoxigenin- labelled DNA probes to denatured metaphase chromosomes and interphase nuclei and visualisation of those probes with fluorochrome conjugated reagents. Minor variations in hybridisation and washing conditions permit successful visualisation of single-copy probe, whole arm paint (CAP), whole chromosome paint probes and repetitive-element probes.

2.4.1 Probe preparation

Different types of X and Y chromosome specific probes were used in this study (Table 2.2). Some of the probes were prepared in this department and some were commercially obtained. Probes used in this study consisted of single-copy probes, repetitive element probes, whole arm paints (CAP), and whole chromosome paints.

Fourteen different probes specific for the X-chromosome were used. A set of three overlapping cosmids CPT4, CAL24 and CPT1, mapping to Xp21.1-21.3 were used. These cosmids cover 80 Kb of the distal half of intron 44, exon 45 and 10 kb of the 5' end intron 45 of the dystrophin gene (Blonden et al, 1989). Another set of three cosmids 141R, G9L and VK21, mapping to Xq27-28 of the FRAX-A/E gene (fragile site) were used. These cosmids correspond to the loci G9L (G9LB, 1CRFC 104DO8104), 141R (EZ165, 1CRFC 100E2165) and VK21 (G3/1) respectively (Hirst et al, 1991, Flynn et al, 1993). The G9L locus lies approximately 70 Kb proximal, 141R 120 Kb distal and VK21 up to 650 Kb distal to the FMR1 CpG island (Flynn et al, 1993).

The commercial probes that were used in this study were DXZ1, KAL, DXZ4, XIST and X-chromosome paint. The DXZ1 (Oncor) is a 2.0 Kb BamHI fragment which hybridises to highly repeated alphoid DNA located at the centromere of the X-chromosome (Waye and Willard, 1985). Both biotin and digoxigenin labelled DXZ1 were used. The Kallman (KAL and DXZ1 commercially combined probes from Oncor) allows the identification of deletion in the region of the KAL gene at Xp22.3 region (Ballabio et al, 1989). The DXZ1 control probe is included to facilitate chromosome identification. The DXZ4 (Oncor) is a human X chromosome microsatellite probe which detects satellite DNA at a locus near band Xq24. The Oncor probe (XIST) for the X-inactivation locus was also used in the study for identifying the specific human sequences in the *XIST* (Xi-specific transcript) gene in either metaphase chromosomes or

the *XIST* (Xi-specific transcript) gene in either metaphase chromosomes or interphase nuclei. This probe is assigned to Xq13.2 (Brown et al, 1991). Whole chromosome paint of X chromosome was purchased from Oncor or Cambio and was either biotin or digoxigenin-labelled. Whole arm paints specific for the short arm and long arm of the X chromosome were purchased from Biomedical Research Corp (Arlington, VA, USA).

Y specific probes used in this study were PDP97, hocSRY cosmid probe two cDNA probes for AZF region (MK5 and MK29), PHY2.1, GMG10 and whole Y chromosome library (Y-paint) probe. The PDP97 probe is a sub-clone derived from cosmid Y97 (Wolfe et al, 1985) which at high stringency detects a repeated Y-specific EcoRI fragment (DYZ3), a marker for the centromere of the Y chromosome.

The hocSRY cosmid probe contains a 36 Kb DNA insert from human Yp11 which allows the identification of sex determination region SRY gene (Su and Lau, 1993). The MK5 and MK29 are 1.9 Kb cDNA cosmid probes assigned to human Yq11.2, a region specific for azoospermia factor in male. (Ma et al, 1993). The GMG-10 probes are 6 Kb HindIII fragments which hybridise to repetitive DNA sequences at Yp11.1-11.2 (Affara et al, 1986). The PHY2.1 probe is a 2.45 Kb HaeIII fragment that hybridises to highly repetitive DNA sequences (in ~2000 copies) at the heterochromatic Yq region. Whole chromosome paints specific for chromosome Y (biotinylated) were purchased from Cambio for use in this study (Table 2.3). All cosmid and plasmid probes were amplified by growing in appropriate culture media containing the appropriate antibiotic (100 µg/ml ampicillin or 60 µg/ml kanamycin) and then were used for DNA isolation.

Table 2.2: DNA probes specific for X chromosome used in this study.

| <i>PROBE</i> | <i>VECTOR INFORMATION</i> | <i>MAP LOCATION</i> | <i>REFERENCE</i> |
|--------------|--|-------------------------------|--------------------------------------|
| cPT1 | Cosmid C2RB BamHI site; 100µg Ampicillin | Xp21.1-21.3 DMD locus | Blonden et al 1989 |
| cAL24 | Cosmid C2RB Bam HI site; 100µg Ampicillin | Xp21.1-21.3 DMD locus | Blonden et al 1989 |
| cPT4 | Cosmid C2RB Bam HI site; 100µg Ampicillin | Xp21.1-21.3 DMD locus | Blonden et al 1989 |
| 141R | Cosmid EZ165.ICRFC 100E2165; 60µg Kanamycin | Xq27-28 DXS296 FRAX-A/E | Hirst et al 1991 Flynn at al 1993 |
| VK21 | Cosmid G3/1; 60µg Kanamycin | Xq27-28 DXS296 FRAX-A/E | Hirst et al 1991 Flynn at al 1993 |
| G9L | Cosmid G9LB ICRFC 140D8104; 60µg Kanamycin | Xq27-28 DXS296 FRAX-A/E | Hirst et al 1991 Flynn at al 1993 |

Table 2.3: Y chromosome DNA/cDNA specific probes investigated in this study.

| <i>Probe</i> | <i>Vector</i> | <i>Insert size</i> | <i>Map location</i> | <i>Reference</i> |
|----------------------|---|---|----------------------|-------------------|
| hocSRY | Cosmid Lorist 5 60 µg Kanamycin | 36Kb 0.9Kb <i>HincII</i> or 12.6Kb <i>EcoR</i> I or 2.1Kb <i>Hind</i> II | Yp11.1 SRY locus | Su and Lau 1993 |
| MK5 CDNA | Cosmid A5F 100µg Ampicillin | 1.9 <i>EcoR</i> I | Yq11.2 RBM1 locus | Ma et al 1993 |
| MK29 CDNA | Cosmid A5F 100µg Ampicillin | 1.9 <i>EcoR</i> I | Yq11.2 RBM1 locus | Ma et al 1993 |
| GMGY10 | Chorion 21 HindIII 100µg ampicillin | 6Kb <i>Hind</i> III | Yp11.1-11.2 | Affara et al 1989 |
| PHY2.1 | Plasmid PSP65SmaI 100µg Ampicillin | 2.4Kb <i>Hae</i> III | Yq12-qter DYZ2 | Cooke et al 1982 |
| PDP 97 | Cosmid Yq7 60µg Ampicillin | 5.5Kb <i>EcoR</i> I | Centromere DYZ3 | Wolfe et al 1985 |

2.4.2 Transformation and growth of the probe and glycerol stock preparation

Probes *hocSRy* specific for *SRY* gene were kindly provided by Dr. Lu and MK5, MK29 cDNA probes were generously provided by Dr Ann Chandley. These cosmids were transferred to DH5 α TM E.Coli competent cells (Gibco BRL) according to the manufacturers protocol with slight modification. The competent cells were removed from -70°C and thawed on ice. The cells were gently mixed using chilled sterile pipette tips and 50 μ l of cell mix transferred to ice chilled sterile 1.5 ml eppendorf tubes. 10 μ l of the cosmid DNA (10 ng) were added to all tubes containing the competent cells except one tube used as a negative control. Tubes were gently shaken for 5 seconds and then incubated on ice for 30 minutes. The cells were then subjected to heat shock in a 42°C water bath for 2 minutes without shaking and rapidly placed on ice for a further 2 minutes. After adding 800 μ l of SOC media to all tubes, the cultures were incubated at 37°C with shaking (100 rpm) for one hour. 200 μ l of the transformed cell culture were spread into LB agar plates containing the appropriate antibiotics and left to stand at room temperature until the excess moisture had been absorbed. The plates then were incubated at 37°C for 12-16 hours and transferred to 4°C for up to one month. Single colonies were observed in all plates except the negative control. Once transformation occurred, a single colony of each probe was picked by sterile loop and cultured in 5 ml of LB media with the appropriate antibiotic and grown for 16 hours at 37°C with shaking at 225 rpm. The overnight culture was then used for DNA isolation and glycerol stock formation. Glycerol stock was made by mixing 0.7 ml of the bacterial growth with 0.3 ml sterile glycerol, and was then stored at -70°C till use.

2.4.2.1 Growth of plasmid/cosmid probes

Glycerol stocks of transformed bacterial cells containing the probes used in this study were grown to amplify the required DNA probe.

50 µl of bacterial stock was cultured in 50 ml LB media containing the appropriate antibiotic at 37°C with vigorous shaking at 225 rpm for 16 hours. The cultures then were used for the extraction of recombinant plasmid/cosmid DNA.

2.4.2.2 Isolation of recombinant plasmid/cosmid DNA using the CIRCLEPREP kit

Probe isolation was performed according to Circle prep protocol with slight modification. On the day following the culture, the cells were transferred to 50 ml centrifuge tubes and centrifuged at 10,000 rpm (12,000g) for 5 minutes at 4°C in Beckman XL-90 centrifuge using JA-20 rotor (Beckman, California). The supernatant was discarded and the tubes were drained for 1-2 minutes. Then cell pellets were resuspended and mixed well in 4ml prelysis buffer to obtain homogeneous suspension. 4 ml of alkaline lysis reagent was added and mixed for 2 minutes until all the cells were lysed and cell suspension became viscous. Then 4 ml of neutralising solution was added and mixed thoroughly for 1-5 minutes until the solution showed two phases, consisting of a white precipitate suspended in clear liquid of water-like viscosity. The mixture was centrifuged 12,000 rpm (17,000g) for 5 minutes at 4°C. Carefully the supernatant was transferred through a sieve to a new centrifuge tube. Equal volume of isopropanol (approximately 12 ml) was added, mixed well until a fine precipitate formed and then centrifuged at 12,000 rpm (17,000g) for 5 minutes at 4°C. The supernatant was removed and the pellet was drained. The cell pellet was dissolved in 0.5 ml sterile water and the cleared supernatant was transferred to a microcentrifuge tube. At this stage the supernatant could be stored at 4°C or frozen. This cleared supernatant consisted of RNA, cosmid/plasmid DNA and some cellular chromosomal DNA.

To denature the linear cellular DNA without nicking supercoiled plasmid/cosmid DNA, the cloned supernatant was incubated in a boiling water bath for 5 minutes and then the tube quenched immediately on ice water bath for one minute. 300 μ l of LiCl (Lithium chloride) was added to the tube, mixed well and allowed to stand at room temperature for 5 minutes. The mixture then was centrifuged for 30 seconds at 14,000 rpm (12,000g) in Beckman microfuge E (Beckman, California) to pellet down the ribosomal RNA (rRNA) and single-strand DNA (ssDNA). The supernatant was transferred to a new microcentrifuge tube, 600 μ l of isopropanol was added, mixed thoroughly and then centrifuged at full speed for 1 minute. The aqueous phase was discarded, the pellet was drained and then dissolved in 0.5 ml sterile distilled water. Then 300 μ l of LiCl was added and mixed well. To the mixture 75 μ l of circle prep Glass milk was added and then incubated for 5 minutes at room temperature with occasional mixing. The mixture was centrifuged for a few seconds to pellet down circle prep Glass milk/DNA complex, the supernatant was removed and the pellet was washed twice with 1 ml wash solution. After a second wash the tube was spun again for a few seconds and traces of liquid from the bottom of the tube were carefully removed. The cosmid/Plasmid DNA was eluted from the glass milk by resuspending the pellet in 100 μ l sterile distilled water and incubating for 5 minute in 65°C water bath. After incubation the mixture was centrifuged for 1 minute at 14,000 rpm (12,000g) to form a tight pellet of glass milk and a clear supernatant containing cosmid/plasmid DNA which was transferred to a new microcentrifuge tube. A second elution would probably yield an additional 10-20% cosmid/plasmid DNA.

The concentration of DNA of each cosmid/plasmid DNA was estimated using a spectrophotometer at wave length 260 nm. 10 μ l of purified DNA was diluted in 990 μ l distilled water (1:100 dilution fraction) and optical density (O.D) was measured at 260 nm.

Because 1 O.D. at 260 unit corresponds to 50 μ g of DNA per ml and the DNA was diluted by 100, the probe concentration was calculated using the following formula: Probe concentration = OD 260 X dilution factor X 50 = μ g/ml.

2.4.3 Restriction enzyme to release probe insert

To ensure that transformed bacterial cells contained the inserted DNA, enzymatic digestion of the purified DNA was carried out to release the inserted probes. 14 µl of cosmid/plasmid DNA (7-10 µg) was digested in a 50 µl reaction volume using 4 µl of *EcoRI* (for MK5 and MK 29) or 5µl *HincII* (for SRY cosmid DNA), 5 µl of 10X enzyme buffer and 27µl ddH₂O. The mixture was incubated at 37°C for 2 hours. The digest was loaded alongside 1 Kb ladder (Gibco BRL) on 1% low melting agarose gel (Seakem agarose, Fmc bioproducts) in 1 X TAE buffer and electrophoresed at 100 volts for 2-3 hours. The gel was visualised on a UV transilluminator (212nm) and 1.9 Kb or (0.9 Kb) bands were detected corresponding to the probe size of each MK5 and MK29 or SRY respectively.

2.4.4 Probe labelling

Probes are labelled with reporter molecules and fragmented into 200-500 bp fragment, a size that maximises specific hybridisation and decreases fluorescence background. Typical reporter molecules include biotin, digoxigenin and dinitrophenyl (DNP), these reporter molecules are incorporated as labelled nucleotides using nick translation.

In this study labelling of cosmid/plasmid probes was done by nick translation with biotin-11-dUTP (Sigma) and/or digoxgenin labelling 11-dUTP (Sigma) according to the BRL Nick translation Kit protocol with some modification.

(A) Biotin-labelling by nick translation:

To 1 µg DNA, the following solutions were added sequentially in 1.5 ml microcentrifuge tube, 5 µl of solution A4, 2.5 µl Biotin-11-dUTP, solution E up to 45 µl and 5 µl of solution. The mixture was incubated for 90 minutes at 15°C. At

the end of labelling the reaction was stopped by adding 5 µl of solution D. The probe was then precipitated by adding 4.6 µl of 3 M sodium acetate pH 5.2, 1 µl of glycogen, 122 µl iced ethanol, mixed well by vortexing and then centrifuged at 12,000 rpm for 30 minutes. The supernatant was removed and the pellet washed with 75% alcohol and then vacuum dried. The probe was dissolved in 10 µl TE buffer giving a concentration of 100 ng/µl. The mixture was vortexed, briefly centrifuged and kept at room temperature for 2 hours with occasional mixing to ensure complete dissolving of the DNA. The probe was stored at -20°C until used.

(B) Digoxigenin-labelling by nick translation:

The digoxigenin labelling of probes was performed using digoxigenin DNA labelling mixture and a nick translation Kit (Gibco BRL). The labelling was carried out in the same way as biotin labelling except that to 1 µg DNA, 2 µl of digoxigenin-labelling dNTPs, solution E up to 45 µl and 5 µl of enzyme mixture (Solution C, Nick translation kit) were added.

2.4.5 Slide preparation

The quality of the slide is one of the most important factors affecting the degree of hybridisation. Metaphase chromosome spreads were prepared by dropping 3-4 drops of fixed cell suspension on to cleaned, wet slides and left to air dry. Slides were evaluated for quality by using phase contrast microscope. The slides were fixed for one hour in 3:1 methanol: acetic acid fixative, allowed to air dry and then dehydrated through an ethanol series (50%, 70%, 90% 100%) 2 minutes each. The slides were aged by overnight incubation at 42°C or at room temperature. Alternatively slides were pre-treated before hybridisation in 2X SSC pH 7.0 at 37°C for 30 minutes. Treatment in 2XSSC artificially ages the chromosome, making them less sensitive to over denaturation. However the treatment was not necessary if the slides were more than 2 weeks old. Slides then dehydrated successively in 70%, 90%, 100% ethanol at room temperature for 2 minutes each and allowed to dry.

2.4.6 Uni-colour fluorescence in situ hybridisation

To detect a single target on chromosome metaphases, uni-colour fluorescence in situ hybridisation was performed. Both biotin and digoxigenin labelled probes were applied in this project. The overall procedure of pre-hybridisation and hybridisation steps is similar for both biotin and digoxigenin labelled probes except for the detection step. The biotin-labelled probes were detected with either rhodamine or fluorescein conjugated avidin whereas the digoxigenin labelled probes were detected with monoclonal anti-digoxigenin labelled with fluorescein or with fluorescent conjugated anti-digoxigenin.

2.4.6.1 Probe mixture preparation and probe denaturation

In principle, suppression using competitive DNA (Blocking DNA) is required for single-copy clones in various vectors (e.g. plasmid, phages, cosmid), probes with interspersed repetitive (IRS) and unique sequences, and whole chromosome paint probes to suppress repetitive sequences in the probe and to prevent non specific hybridisation. For simultaneous hybridisation of two or more probes, 10 µg human Cot-1 DNA or total human genome DNA should be added.

Generally large probes with a large amount of repetitive sequence should be preannealed for a longer time. Moreover, repetitive, alpha-satellite and telomeric probes and most cDNA probes do not require suppression with Cot-1 DNA because Cot-1 DNA contains repetitive DNA sequences.

In this study hybridisation mixtures were prepared with the proper concentration of labelled probes, the desired concentration of competitive DNA's and hybridisation buffer immediately before application to slides (Table 2.4).

Generally, in experiments using a single cosmid/plasmid probe, 50-250 ng labelled DNA (0.5-2.5 µl from 100 ng/µl stock) 10 µg sonicated human placental DNA (1 µl from 10 mg/ml stock solution) and 0.8 µg sonicated salmon sperm DNA (0.8 µl from 10 mg/ml stock solution) were added to 8.9 µl hybridisation

buffer. In experiments using 3 cosmids together as with the DMD probes and fragile X probes, 100 ng of each labelled probe were used.

All prepared probes were denatured at 72°C for 10 minutes to denature DNA. If the probes did not require suppression, the denatured probe(s) placed on ice. If suppression was required, the denatured probe(s) placed at 37°C and pre-annealed 60 minutes to several hours (Table 2.4 and 2.5).

When co-hybridisation with X centromeric probe was performed 1 µl of DXZ1 (0.5 µg/ 50 µl) was denatured in 10 µl (1 ng/µl) hybridisation buffer, cooled on ice and 0.5 µl of it was combined with pre-annealed probes just before application to slides.

When commercial probes XIST and KAL (Oncor) were used, the experiments were carried out according to the supplier's protocols. The probes were provided premixed with blocking DNA and suspended in Hybrisol VII (50% formamide 2 x SSC). The probes were pre-warmed at 37°C for 5 minutes, gently vortexed and centrifuged 2-3 seconds to collect the contents in the bottom of the tube. Then 10 µl of probe solutions were applied to denatured slides.

Microsatellite DXZ4 probe was prepared according to Oncor protocol as well. This probe was provided with hybrisol V1 (65% formamide, 2 X SSC). The probe was pre-warmed at 37°C for 5 minutes, vortexed and centrifuged for 2-3 seconds. 31.5 µl of probe was placed in 0.5 ml microcentrifuge tube and denatured by heating in 70°C water bath for 5 minutes. The probe was then placed in 4°C ice bath until ready to hybridise.

Total chromosome (Coatasome or Cambio paint) probes were also prepared according to Oncor or Cambio protocol. Specific paints were pre-warmed at 37°C for 5 minutes, gently vortexed and centrifuged 2-3 seconds. 10 µl of probe solution was placed in 0.5 ml microcentrifuge tube. This probe was provided with blocking DNA in hybrisol VII (50% formamide, 2 X SSC). The probe was denatured by heating in 70°C water bath for 10 minutes and then incubated at 37°C for 1 to 2.5 hours to preanneal the DNA.

Table 2.4: Preparation of hybridisation mixture of probes specific for X and Y chromosomes in the uni-colour fluorescence *in situ* hybridisation experiments.

| <i>Biotin-labelled Probe</i> | <i>Volume of DNA used (μl)</i> | <i>Volume of Human Placental DNA (μl)</i> | <i>Hybridisation buffer (12μl total volume) / 22x22 mm area</i> | <i>Pre-annealing Time</i> |
|------------------------------|--------------------------------|---|---|---------------------------|
| DXZ1 (1ng/μl) | 0.5 | - | 11.5* | - |
| PDP97 (100 ng/μl) | 0.5 | - | 11.5* | - |
| CPT-1 (100 ng/μl) | 2.5 | 0.6 | 8.9 | 1 hour |
| CPT-4 (100 ng/μl) | 3 | 0.6 | 8.4 | 1 hour |
| CAL-24 (100ng/μl) | 3 | 0.6 | 8.4 | 1 hour |
| 141R (100 ng/μl) | 2.5 | 0.6 | 8.9 | 1 hour |
| VK21 (100 ng/μl) | 3 | 0.6 | 8.4 | 1 hour |
| G9L (100 ng/μl) | 3 | 0.6 | 8.4 | 1 hour |
| GMGY10 (10ng/μl) | 1 | - | 11* | - |
| PHY2.1 (100 ng/μl) | 1 | - | 11* | - |
| HocSRY (100 ng/μl) | 2.5 | 0.6 | 8.9 | 1 hour |
| MK5 (100 ng/μl) | 2 | 0.5 | 9.5 | 1 hour |
| MK29 (100 ng/μl) | 2 | 0.5 | 9.5 | 1 hour |

* Used hybridisation buffer 1.

NOTE : Hybridization temperature 37⁰ C - 42⁰ C; hybridization time 16 hr.

Table 2.5: Commercial probes used in this study.

| Probe | Source | Amount/25x25mm area | Pre-annealing Time | Annealing Temp/ 16 h |
|---|------------|---------------------|--------------------|----------------------|
| Whole X chromosome paint (WCPX) | Oncor | 10 µl | 2.5 hour | 37° C |
| | Cambio | 10 µl | 1 hour | 37° C |
| Whole Y chromosome paint (WCPY) | Cambio | 10 µl | 1 hour | 37° C |
| XIST (Xq13) | Oncor | 10 µl | - | 37-39° C |
| KAL (Xp22) | Oncor | 10 µl | - | 37° C |
| DXZ4 (Xq24) | Oncor | 31.5 µl | - | 37° C |
| Xq/Yq; Xq28-qter; Yq12-qter (telomeric) | Oncor | 10 µl | - | 37° C |
| Xpter (telomeric) | Oncor | 10 µl | - | 37° C |
| X Chromosome short arm paint (CAP-Xp) | Biomedical | 10 µl | - | 37° C |
| X Chromosome long arm paint (CAP-Xq) | Biomedical | 10 µl | - | 37° C |

2.4.6.2 Cellular DNA denaturation and probe hybridisation

The target DNA was denatured by soaking slides containing metaphase chromosomes 2 minutes in 70°C denaturing solution (70% formamide/ 2 X SSC) in a Coplin Jar in a 72°C water bath. Slides were rinsed for 2 minutes in ice-cold 70% ethanol in coplin jar to stop denaturation. Then continued dehydration by incubating slides 2 minutes each in room temperature 80%, 90%, and 100% ethanol and allowed to air dry maximally up to 15 minutes. Then 10-12 µl or 31.5 µl of preannealed probe mix was added to slides and covered by 22 X 22 or 22 X 50 mm glass cover slip respectively, sealed with rubber cement and incubated overnight (16-20 hrs) in moist chamber at 37-42°C water bath (Table 2.4 and 2.5).

2.4.6.3 Post hybridisation washes

Prior to detection, post hybridisation washes were performed to remove non-specifically bound and weakly hybridised probes. During last 30 minutes of hybridisation, 50 ml of 50% formamide/2X SSC wash and 50 ml 2 X SSC in coplin jars were warmed in 42°C water bath. The slides were removed from moist chamber, the rubber cement peeled off and the cover slips carefully removed. Slides were rinsed in 2X SSC at 42°C for 5 minutes. The slides then washed twice in 50% formamide/ 2 X SSC and twice in 2 x SSC at 42°C for 5 minutes followed by incubation in wash-A solution for 30 minutes at 37°C.

2.4.6.4 Amplification of hybridisation signals

Signals of both biotin and digoxigenin-labelled probes were amplified after post hybridisation washes.

a) Amplification of biotinylation signals:

Slides hybridised with biotinylated probes exposed to avidin were incubated with biotin-labelled antibody to avidin. Since every avidin molecule

contains four biotin-binding sites, biotin-labelled antibody to avidin can potentially bind the avidin molecule by a biotin-avidin interaction or an antibody interaction. A final amplification of fluorescence-conjugated avidin results in significant signal amplification. Detection of biotinylated signals was performed as follows: 100 μ l of 5 μ g/ml FITC conjugated avidin (diluted in wash-A) or 100 μ l of 5 μ g/ml rhodamine-avidin (prepared in wash A solution) were applied to each slide, covered with parafilm, and incubated at 37°C for 15-20 minutes. Slides were rinsed 3 X 5 minutes in wash-A at 42°C. Then 100 μ l of 5 μ g/ml biotinylated anti-avidin (diluted in wash A) was added to each slide, covered with parafilm, and incubated at room temperature. For FITC conjugated avidin detection system a final amplification was carried out by adding 100 μ l of 5 μ g/ml FITC conjugated avidin (diluted in wash A) and incubated for 15-20 minutes at room temperature. Finally, slides were rinsed 2X5 minutes at 42°C in wash-A and 2X5 minutes in 4 X SSC, 0.5% Tween-20 at room temperature, dehydrated through an ethanol series 50%, 70%, 90%, 100% 2 minutes each and allowed to air dry.

b) Amplification of signals from digoxigenin-labelled probes:

Fluorescence signal for digoxigenin-labelled probes is achieved by sandwiching a non-fluorescent fragment of sheep anti-digoxigenin between the digoxigenin labelled probe and a fluorochrome-labelled anti-sheep IgG. Since more than one Fab fragment can attach to a single digoxigenin molecule and each of the Fab fragments is recognised by the fluorochrome-conjugated second antibody, significant signal amplification occurs. The digoxigenin-labelled probes were detected as follows: 100 μ l of 0.6 μ g/ml anti-digoxigenin fluorescein Fab fragment prepared in sheep (prepared in wash-A solution) was applied to the hybridised area, covered with parafilm and incubated at 37°C for 15-20 minutes. Slides were rinsed 3X5 minutes in 42°C wash A and incubated with 100 μ l of 2.25 μ g/ml fluorescent anti-sheep IgG for 15-20 minutes at room temperature. Slides were washed 2X5 minutes in 42°C wash-A solution, 2X5 minutes in 4 X SSC/ 0.5% Tween-20 at room temperature, dehydrated through an ethanol series 50%, 70% 90% and 100% two minutes each and then air dried.

2.4.6.5 Chromosome counter staining

Chromosomes were stained by adding 10-12 μ l of the appropriate counter stain. When probe was detected by FITC-avidin (green), chromosomes were counter stained using both DAPI and propidium iodide. 1 μ l of DAPI (40 μ G/ML) and 2 μ L of propidium iodine (20 μ g/ml) were diluted in 97 μ l of antifade mounting medium AF1 (Citifluor Ltd), slides were covered with 22 m² coverslip and sealed with nail polish. Slides were stored in the dark at -20°C till scored.

When rhodamine (red) was used, chromosomes were detected with counter stain containing only DAPI. 1 μ l of DAPI (40 μ g/ml) was diluted in 99 μ l of antifade solution.

2.4.7 Multi-colour FISH

A dual colour and a three colour combinatorial mixing FISH method was applied to metaphase chromosomes to detect simultaneously the probe sets specific for X chromosome or Y chromosome. A dual colour detection protocol can be used for two probes (one labelled with biotin and one with digoxigenin) but a third probe labelled with both can also be recognised. In dual colour the hybridisation mixture contained two specific probes of which one was labelled with biotin-11-dUTP and the other with digoxigenin-11-dUTP. When ratio-mixing FISH was applied a third probe was labelled with either biotin 11-dUTP or digoxigenin 11-dUTP and mixed in a 1:1 combination (Table 2.6).

2.4.8 Detection and counter staining of multi-colour FISH

The pre-hybridisation, hybridisation and post hybridisation washing steps for multi-colour FISH were performed as for uni-colour FISH except for detection step. In all multi-colour FISH studies the biotin-labelled probes were detected with avidin conjugated with rhodamine and the signals were amplified by using biotinylated anti-avidin and adding a second layer of avidin conjugated with rhodamine.

Table 2.6: Hybridisation mixtures applied in the dual and in the combinatorial FISH studies.

| Biotin probe | Digoxigenin Probe | Human placental DNA | Hybridisation Buffer | Pre-annealing Time |
|---------------------------|------------------------------|---------------------|----------------------|--------------------|
| DXZ1=1 µl | DMD = 3µl* | 1 µl | 9 µl | 1 h |
| DXZ1=1 µl | FRAXA/E*= 3 µl | 1µl | 9 µl | 1 h |
| KAL =10 µl | DXZ1 =1µl | - | - | - |
| DXZ1 =1 µl | XIST =10 µl | - | - | - |
| DXZ1 =1 µl | DXZ4 =31.5 µl | - | - | - |
| PDP97 =1 µl | DXZ1 = 1 µl | - | 10µl | - |
| DMD = 3 µl* | XIST = 10 µl | - | - | - |
| KAL = 10 µl | DXZ = 31.5µl | - | - | - |
| DMD =2.5 µl | FRAXA/E= 2.5µl | 1 µl | 8µl | - |
| MK5 = 2.5 µl* | PDP97 = 1µl | 0.6 µl | 9 µl | - |
| hocSRY = 2.5 µl* | PDP97 = 1µl | 0.6 µl | 9µl | - |
| MK29 = 2.5 µl* | PDP97 = 1µl | 0.6 µl | 9 µl | - |
| GMGY10 = 1 µl | PHY2.1 = 1 µl | - | 10µl | - |
| hocSRY = 2.5 µl* | DXZ1 = 1 µl | 0.6µl | 9µl | - |
| hocSRY = 2.5µl | MK5 = 2 µl | 0.6µl | 9µl | 1 h |
| DXZ1= 1µl + KAL = 10 µl | DXZ1 = 1 µl | - | - | - |
| DXZ1 = 1 µl | DXZ1 = 1 µl + XIST = 10 µl | - | - | - |
| DXZ1 = 1 µl | DXZ1 = 1 µl + DXZ4 = 31.5 µl | - | - | - |
| DXZ1 = 1 µl + DMD = 3 µl | DXZ1 = 1 µl + FRAX = 3 µl | 1 µl | 8 µl | 1 h |
| DXZ1 = 1 µl + KAL = 10 µl | DXZ1 = 1 µl + XIST = 10 µl | - | - | - |
| DXZ1 = 1 µl | DXZ1 = 1 µl + DXZ4 = 31.5 µl | - | - | - |

*The probe first pre-annealed to the competitor DNA and then the second probe was added to the mixture before application to the slide.

Note: Hybridization temperature was at 39^oC to 42^oC for 16 hour.

The digoxigenin-labelled probes were detected by FITC conjugated monoclonal anti mouse IgG. The detection method was as follows: 100 µl of 5 µg/ml FITC conjugated avidin was applied to the hybridised area, covered with parafilm and incubated at 37°C for 15-20 minutes. Slides were rinsed 3X5 minutes in 42°C wash-A. 100 µl of mixture of 5 µg/ml of goat anti-avidin D and 5 µg/ml of FITC conjugated monoclonal anti-digoxin prepared in mouse (diluted in wash-A) was added to the slide, covered with parafilm and incubated at room temperature for 15-20 minutes. Slides were washed 2X5 minutes in wash-A at 42°C and then incubated at room temperature for 15-20 minutes with 100 µl of a mixture of rhodamine avidin (5 µg/ml) and FITC conjugated anti-mouse IgG (5 µg/ml prepared in wash-A solution). Slides were washed 2X5 minutes in 42°C wash-A and 2X5 minutes in 4 X SSC/0.5% Tween-20 at room temperature, dehydrated through ethanol series 50%, 70%, 90% and 100% and air dried. Slides were counter stained and mounted in DAPI/antifade stain as previously mentioned.

2.4.9 Visualisation

All FISH studies were examined and analysed using a fluorescence microscope with epi-illumination equipped with filter sets appropriate for the fluorochrome used (Zeiss Axioplan) (Table 2.7). A DAPI filter set was used for chromosome identification and a triple-band-pass filter set was used for simultaneous viewing of multiple fluorochrome. The epifluorescence microscope also equipped with a Photometric charge coupled device (CCD) camera and computer software (SmartCapture; Digital Scientific). The images were captured and printed on sublimation printing paper (A4-SPW) using a Mitsubishi colour printer.

Table 2.7: Filters applied to visualize fluorochromes by epifluorescence microscopy.

| <i>Fluorochrome</i> | <i>Excitation Wavelength (nm)</i> | <i>Emission Wavelength (nm)</i> | <i>Zeiss - Filter</i> | <i>Colour of Fluorescent</i> |
|-----------------------------|---------------------------------------|-------------------------------------|---------------------------|----------------------------------|
| DAPI | 345 | 425 | 1,2 | Blue |
| Propidium iodide | 520 | 610 | 9 | Red |
| FITC | 490 | 525 | 9,10 | Green |
| Rhodamine | 540-560 | 580 | 15 | Red |

2.5 Molecular Genetics study

2.5.1 DNA extraction

DNA was extracted from whole blood following the protocol described by Kunkel et al (1977). 5-10 ml EDTA of blood were transferred to a falcon tube and lysis buffer (0.32 M sucrose, 10 mM Tris-Cl pH 7.5, 5 mM MgCl₂, 1% Triton 100) was added up to 50 ml. The samples were mixed and centrifuged in Beckman GS-6 centrifuge using GH-3.7 rotor (Beckman, California) at 2,500 rpm (1,430g) for 10 minutes at 4°C. The supernatant discarded into a beaker containing 5% Chloros and the pellet resuspended in 3 ml nuclei mix (10 mM Tris, 0.4 M NaCl, 2 mM EDTA, pH 8.2. 200 µl 10% SDS (Sodium Dodecyl Sulphate) and 100 µl of protease K (10 mg/ml) were added. The mixture then vortexed thoroughly and incubated at 37°C for 16 hr. 1 ml of 6M NaCl was added and the samples were shaken vigorously, and were centrifuged at 3,000 rpm (2,060g) for 15 minutes. The top layer was carefully transferred into a clean centrifuge tube and 800 µl phenol/chloroform added and mixed by inversion several times for 1 minute. The tube was then centrifuged at 2,500 rpm (1,430g) for 15 minutes. The supernatant was then transferred into a sterile tube and the DNA was precipitated by the addition of two volumes of 100% ethanol. The DNA was spooled out using a clean sealed Pasteur pipette, air dried and dissolved in 300-500 µl TE buffer (10 mM Tris, 1 mM EDTA pH 7.5). The DNA was left to dissolve overnight at room temperature. The concentration of DNA was calculated by measuring the optical density at 260 nm and the DNA stored at 4°C.

2.5.2 Agarose gel electrophoresis

1% agarose gel was prepared by dissolving 0.5 g agarose powder (Seakem GTG, FMC Bioproduct) in 50 ml 1 X TBE buffer. The mixture was boiled, cooled down 55°C, 1 µl ethidium bromide was added (10 mg/µl) and then allowed to set. Samples were mixed with 1/5 volume of 6 X TBE loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% w/v sucrose in water) were loaded

and the gel electrophoresed in 1 X TBE buffer at 10-15 v/cm until the bromophenol blue in the loading mixture migrated two thirds of the distance of the gel. The DNA was then visualised with a UV light (212 nm) transilluminator and photographed.

2.5.3 Oligonucleotide design and preparation

Oligonucleotides were designed to amplify DNA sequences from both *YRRM* (*RBM-1*) and *SRY* genes (Appendix I Tables II 2 and II7). The computer program OligoTM version 3.4 (Medprobe) was used for primer designing. Primers of 20 to 22 bases were designed to be, as much as possible, free of significant complementarity at their 3' termini, free of self-complementarity and able to form a stable duplex (by calculating the free energy of duplex formation, ΔG) with the specific site only on the target DNA. The T_m (melting temperature) difference between both primers and between the template and the less stable primer is kept to a minimum with avoidance of runs of C's or G's at the 3' ends of the primers.

All primers, either published or newly designed (Appendix I) were synthesised in-house using an ABI 391-DNA synthesiser with "Trityl off" (Applied Biosystem). The end products existed as phosphate-protected, base protected phosphotriesters, therefore, complete deprotection was required to produce active oligonucleotides. The extraction of the primers from the column was carried out as follows:

The primers were cleaved and the cyanoethyl-protecting group (phosphate deprotection) was removed by treating the primers with fresh concentrated ammonium hydroxide for one hour. Base deprotection (ammoniolysis reaction and the removal of benzoyl and isobutyryl base protecting group) was followed by placing the vials contained the collected oligonucleotides in a 55°C water bath for 5-18 hours. The ammonium hydroxide-oligonucleotide solution was cooled at room temperature for at least 30 minutes. Primers were then stored in ammonia eluant at -20°C till needed. Before using, purification of primers from ammonia was performed. Aliquots from the ammonia stock were left in opened tubes under the fume hood for 16-24 hours to ensure complete evaporation of the ammonia.

The concentration of the primers was estimated by measuring the O.D. using UV spectroscopy at 260 nm. Because 1 O.D.₂₆₀ of single-strand oligonucleotide represents approximately 33 microgram of DNA/ml therefore, primer concentrations were calculated as follows: Oligonucleotide concentration = OD₂₆₀ X dilution factor X 33 = µg/ml. The oligonucleotide solution was diluted to 5 µM concentration and stored at -20°C.

2.5.4 Polymerase chain reaction (PCR)

Different sets of primers, both published and in house designed, were used for PCR to amplify exons and regions of interest from different genes [Androgen receptor (*AR*), *SRY*, and *RMB-1*] (Appendix I). All reactions were optimised individually. Each reaction was carried out in 50 µl final volume containing 250 ng of genomic DNA as a template, 30 pmol of the relevant sense and antisense primer, 200 µM of each dNTPs, 5 µl of the 10X PCR buffer (100 mM Tris Hcl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% gelatine) and 2.5 units Taq polymerase. The reactions were mixed and then overlaid with mineral oil. The samples were subjected to 28 cycles of denaturation at 94°C for 1 minute, annealing at the appropriate temperature for each segment for 1 minute and extension at 72°C for 1 minute followed by a final extension step at 72°C for 10 minutes. PCR products were checked by gel electrophoresis on 1% agarose mini-gels with the product being compared against a 100 bp or 1 kb ladder (Gibco BRL).

2.5.5 Multiplex PCR

Multiplex PCR screening methods were performed using 28 primer pairs (Appendix I, Table I-5) in five multiplex PCR experiments. These primer pairs were used to screen for the presence or absence of 28 Y-specific DNA sequences in Yq11 in 56 infertile male patients. The screening method was applied according to Henegariu et al (1994) with some modifications.

Primer start solutions for the multiplex PCR were optimised so that each primer concentration in each multiplex reaction was optimal for the amplification of its corresponding genomic template (Table 2.8).

Table 2.8: Primer volumes for the multiplex PCR experiments.

| MULTIPLEX 1 | | MULTIPLEX 2 | | MULTIPLEX 3 | | MULTIPLEX 4 | | MULTIPLEX 5 | |
|-----------------|-------------------------|-----------------|-------------------------|-----------------|-------------------------|-----------------|-------------------------|-----------------|-------------------------|
| Primer (F/R) | Volume of primer DNA | Primer (F/R) | Volume of primer DNA | Primer (F/R) | Volume of primer DNA | Primer (F/R) | Volume of primer DNA | Primer (F/R) | Volume of primer DNA |
| sY84 | 0.6µl | SY143 | 1µl | SY86 | 0.4µl | sY14 | 1.1µl | Y6BaH34pr | 0.4µl |
| sY134 | 1.5µl | SY157 | 0.5µl | SY105 | 0.4µl | sY95 | 0.8µl | FR15-11pr | 1.2µl |
| sY117 | 0.4µl | sY81 | 0.5µl | SY82 | 0.5µl | sY127 | 1.5µl | Y6Hp52pr | 2.8µl |
| sY102 | 0.35µl | sY182 | 1.2µl | Y6Hp35pr | 0.3µl | sY109 | 0.8µl | Y6Hp35 | 0.7µl |
| sY151 | 2µl | sY147 | 2.2µl | Y6Hc54pr | 0.8µl | sY149 | 0.8µl | Y6D14pr | 0.8µl |
| sY94 | 1µl | | | SY153 | 1.2µl | | | | |
| sY88 | 1.2µl | | | SY97 | 1µl | | | | |

F= Forward primer; R= Reverse primer
1µl = 20 pmol

Each multiplex PCR was performed in 25 µl PCR reaction. To each multiplex primer start solution, 150ng of genomic DNA, 200 µM dNTP mix, 5% DMSO (dimethyl sulfoxide), 2.5µl of 10X PCR buffer, and 2.5 unit of DNA Taq polymerase was added (Table 2.8). The complete PCR mix was overlaid with mineral oil and put directly in a thermocycler preheated to 94°C. The templates were initially denatured at 94°C for 5 minutes followed by 45 cycles at 94°C for 30 seconds (melting); 54°C for 45 seconds (annealing) and 65°C for 120 seconds (extension). A final extension at 65°C for 5 minutes was performed. At the end of PCR amplification the tubes were stored at 4°C or -20°C till use.

2.5.6 Gel analysis of multiplex PCR

The PCR products were separated on 3% agarose gels (Seakem LE quality) by electrophoresis in 1XTAE buffer at room temperature using a voltage gradient of 8 volt/cm. For increasing the sharpness of gel fragments Nuseive:Seakem (3:1) gels in TBE buffer were used. 5µl of 5X loading buffer (15% Ficoll, 0.25% Xylene cyanol) was added to the PCR product and half of the PCR reaction mix was loaded on the gel and electrophoresed for 3 hours at 10 volts/cm then photographed.

2.5.7 Southern blot analysis

(A) Probe preparation

Digests were set up to release the probe inserts MK-5 and MK-29 from cosmid DNA in 50 µl total volume reactions. 14 µl of cosmid DNA, obtained by the CIRCLE PREP kit, 2 µl (50 units) *EcoRI* restriction endonuclease, 10 µl 10X enzyme buffer and ddH₂O up to 50 µl was incubated at 37°C for 2 hours. The digest was electrophoresed alongside a 1Kb ladder (Gibco BRL) on 1% low melting agarose gel (Seakem agarose, FMC bioproducts) for 3-4 hours at 100 volts. The gel was visualised on a UV transilluminator and the 1.9 kb insert band was cut by a sterile scalpel blade and placed in a pre-weighed 1.5 ml tube. The sample was weighed and 2 ml of sterile T.E. buffer was added for every gram of

sample. The insert was then boiled for 5 minutes and stored at -20°C until used.

(B) Southern blotting

7 to 10 μg of genomic DNA was digested using the appropriate restriction endonuclease in a total volume of 50 μl containing 1X appropriate enzyme buffer and 40 units of the specific enzyme. The digests were incubated in a water bath set at the recommended temperature for 16 hours. Digests were stored at -20°C till used.

The digested fragments were separated alongside a 1 kb ladder in 0.8% agarose gels prepared in 1X TAE buffer and containing 0.5 $\mu\text{g/ml}$ ethidium bromide. The gel was electrophoresed at 2 volts/cm for 16-20 hours. At the end of electrophoresis the gel was visualised and photographed alongside a ruler on a UV transilluminator. The gel was then transferred to a plastic box, rinsed several times in water, treated in a depurination solution (0.2M HCl) for 15-20 minutes then rinsed in water. The gel was then treated with the denaturation solution (0.5M NaOH, 1.5M Na Cl) for 30 minutes, rinsed in water and neutralised in two changes of neutralisation solution (3M NaCl, 0.5M Tris pH 7.4) for 15 minutes each.

The blotting apparatus was set up by placing a glass plate across a plastic box contained 10X SSC. The glass plate was covered by a double layer of 3MM Whatman paper with its ends dipped into the SSC. Any air bubbles between the paper and the platform was smoothed out. The treated gel was placed onto the wick making sure that there were no air bubbles between the gel and the 3MM paper. The gel was surrounded by a plastic wrap, to ensure that buffer would proceed only through the gel. A sheet of Hybond N membrane was cut to size, then layered over the gel avoiding air bubbles. Two layers of 3MM paper soaked in 2X SSC were then placed on to the membrane and air bubbles were removed. A stack of paper towels (~ 10 cm high) was placed on top of the filter paper. A glass plate and a weight of ~ 500 g were laid on top of the absorbing paper stack. The blotting was carried out overnight. The next day the blot was dismantled and the filter was baked for 4 hours in an 80°C oven to fix the DNA.

(C) Hybridisation of DNA blot

Prehybridisation of filter

The filter was placed, in the hybridisation bottle, with 10 ml of the prehybridisation solution (6X SSC, 0.5% SDS, 5X Denhardts) and 10 μ l boiled sonicated salmon sperm DNA (10 mg/ml) and incubated in an 65°C hybridisation oven overnight.

Probe labelling and filter hybridisation

The probe was radioactively labelled using the random primed DNA labelling kit (Boehringer Mannheim) according to the supplier's protocol. 24 μ l of the probe (in low melting agarose) were boiled for 10 minutes, then incubated at 37°C for one minute. The probe was then added to a mix of 2 μ l each of dATP, dGTP and dTTP, 4 μ l of the reaction mix, 1.5 μ l of Klenow enzyme and 5 μ l (50 μ Ci) of α -³²P dCTP (Amersham). The tube was then incubated at 37°C for 2.5 hours. The labelled probe was then separated from the unincorporated α -³²P dCTP using G-50 sephadex NICKTM columns (Pharmacia Biotech.). The column was washed with 3 ml 1X SSC. The labelled probe (~ 40 μ l) was added to the top of the column and then 400 μ l 1X SSC was added and allowed to drip through. A further 400 μ l 1X SSC was then added to the column and the drops were collected.

The labelled probe was boiled for 4 minutes and added to the prehybridisation solution. The filter was left to hybridise overnight at 65°C. After hybridisation, the hybridisation solution was discarded and the filter was washed in 2X SSC/0.1% SDS for 10 minutes at 65°C. The filter was removed, monitored and placed in a plastic box with 0.5X SSC/0.1% SDS and washed by shaking at 65°C in a water bath for 5 to 10 minutes. If a signal greater than 10 cpm (count per minute) was detected, the washing was continued with increasingly stringent solutions (65°C, 0.25X SSC/0.1% SDS then 0.1X SSC/0.1% SDS) until the filter had washed down to less than 5 cpm.

(D) Autoradiography

The filter placed in an autoradiographic cassette with intensifying screens and exposed to Kodak Diagnostic AR Imaging film. The cassette was stored in a -40°C freezer for at least one week. The autoradiograph was then developed and analysed.

2.5.8 Single strand conformational polymorphism (SSCP) analysis

SSCP analysis was applied to screen for point mutations using MDE™ gels (mutation detection enhancement), which significantly improve the resolution of DNA molecules with different conformations.

Radiolabelled PCR amplifications of exons and regions of interest from different genes were performed. PCR amplifications were done using the standard PCR protocol. 15 µl PCR reaction mix was prepared to contain 1X GeneAmp PCR buffer, 100 µM of each dNTP, 1.5 pmol of each primer, 100 ng genomic DNA, 2 µCi α -³²P dCTP and 2 units AmpliTaq polymerase. The reaction was overlaid with 25 µl mineral oil and was subjected to thermal cycling with the appropriate annealing temperature for each specific exon or segment (Appendix I). All segments of the *SRY* gene, exons 2-7 of the *AR* gene and exons 1-11 of the *RBM1* gene were screened by SSCP analysis.

At the end of thermal cycling the amplified products were diluted 1:9 in loading buffer (95% formamide, 10mM NaOH, 0.5% bromophenol blue and 0.5% xylene cyanol), heat denatured at 95°C for 3 minutes then quenched on ice for at least 5 minutes before loading to SSCP gel. 3 µl of each sample was loaded on 0.5X MDE non-denaturing gel containing 5% glycerol. The gel was electrophoresed in 0.6X TBE buffer at 5 watts for 16 hours at room temperature.

After gel electrophoresis, the glass plates were separated gently and the gel was transferred to 3MM Whatman paper and covered with Saran wrap. The gel was dried under vacuum for 45 minutes at 80°C, with the Saran wrap upper most on a gel dryer (Biorad, Model 583).

Autoradiography was carried out by placing the dried gel with the DNA

side facing the X-Omat diagnostic AR film (Kodak) in an autoradiographic cassette with intensifying screens. The gel was autoradiographed for 5 to 16 hours at -40°C.

2.5.9 Heteroduplex analysis

This method was based on the protocol described by Keen et al (1991) with some modifications. Non-radiolabelled PCR amplification was done using the standard protocol as previously mentioned. At the end of the PCR cycles the reaction was inactivated by adding EDTA to a final concentration of 5mM. PCR products of a test sample and a wild type control were mixed, heated to 95 °C for 5 minutes and cooled slowly to room temperature over 20 to 30 minutes to form any heteroduplexes. Meanwhile, a 50 cm, 1 mm thick, 1X MDE gel was prepared in 1X TBE buffer and allowed to set for at least 1 hour. Samples were prepared by adding 10µl of 6X formamide loading buffer (95% deionized formamide, 10mM EDTA, 10 mg/ml bromophenol blue and 10 mg/ml xylene cyanol) for every 5 µl PCR product. 5-10 µl of samples were loaded and electrophoresed in 0.6X TBE buffer alongside a 1kb ladder and a positive control heteroduplex sample at a constant 800 volts for 20-24 hours. The gel was analysed by silver staining and photography. This technique was applied to exon 8 of the *AR* gene and exon 12 of the *RBM1* gene.

2.5.10 Silver staining

The gel was first fixed in two changes of fixation solution (10% ethanol, 0.5% acetic acid) each for 5 minutes with gentle shaking. Then, it was stained for 15 minutes in freshly prepared 0.1% silver nitrate, rinsed briefly with distilled water, incubated in developing solution (1.5% NaOH, 0.1% formaldehyde mixed just before use) for 30 minutes, treated with 0.75% Na₂CO₃ solution for 10 minutes, sealed in a plastic bag and photographed.

2.5.11 Chemical cleavage of mismatches (CCM)

Chemical cleavage of mismatches analysis was applied as a part of the mutation detection strategy. The technique was performed according to Cotton et al (1989), with slight modifications.

(A) Preparation of non-radiolabelled test DNA and radiolabelled probe

The open reading frame of the *SRY* gene was amplified by PCR using XE10/XE11 primers (Appendix I-2) from four patients with sex reversal and gonadal dysgenesis following the standard protocol, in a total volume of 50 μ l. A radiolabelled PCR product (probe) of the same segment from a normal control male DNA was also amplified by direct incorporation of 2 μ Ci of α -³²PdCTP in the PCR reaction mix. 5 μ l of the PCR products were electrophoresed in a 1% agarose gel containing 0.5 μ g/ml ethidium bromide and visualised with a UV transilluminator to confirm the specificity of the PCR. The remaining 45 μ l PCR products were then cleaned using GenecleanTM purification kit (Bio 101 Inc.) following the supplier protocol.

(B) Formation of heteroduplexes

Hybridisation between probe and target DNA was set up in 10-20 fold molar excess of target DNA over the probe DNA. For each modification reaction approximately 5-10 ng of probe DNA was hybridised with 100 ng test DNA in a mixture containing 1X hybridisation buffer (0.3M NaCl; 0.1M Tris-HCl, PH 8.0) in T_{0.1}E buffer (10mM Tris-HCl, PH 7.4; 0.1mM EDTA). The reaction was layered with an equal volume of mineral oil in 0.5 ml Eppendorf tube, boiled for 5 minutes and then incubated in a 65°C water bath for 5-16 hours. The reaction mixture was then equally divided into two 1.5 ml siliconised Eppendorf tubes. 750 μ l of stop/precipitation mixture (63 mM Na acetate; 20 μ M EDTA; 80% ethanol) and 3 μ l of muscle glycogen (20mg/ml) were added to each tube, mixed well and kept on dry ice for 10 minutes. Tubes were then centrifuged in a microcentrifuge (Beckman, California) at 14,000 rpm (12,000g) for 10 minutes. The pellets were washed in 70% ethanol, air dried and resuspended in 7 μ l T_{0.1}E

buffer. Tubes can be stored up to 24 hours at -20°C before being used in the next step.

(C) Chemical modification with osmium tetroxide and hydroxylamine

A mixture of osmium tetroxide and pyridine was freshly made on ice by mixing 6.75 μl pyridine with 1.5 μl of 4% osmium tetroxide and 154 μl T_{0.1}E buffer. To each 7 μl hybrid mixture, 18 μl of osmium/pyridine mixture were added, mixed and the tubes incubated for 2 hours at 37°C . Meanwhile, 1.39 grams of hydroxylamine was dissolved in 1.6 ml of water and the pH was adjusted to 6 by adding approximately 1.5 ml of diethylamine. 20 μl of this solution was mixed with 7 μl of the hybrid mixture and the reaction was then carried out at 37°C for two hours. The modified reactions were stopped and precipitated by mixing 750 μl of stop/precipitation (63 mM Na acetate, 20 μM EDTA, 80% ethanol) solution with each reaction. Tubes were incubated on dry ice for 10 minutes and then centrifuged at 14,000 rpm (12,000g) for 10 minutes in microcentrifuge (Beckman, California). The pellets were washed in 70% ethanol and air dried.

(D) Piperidine cleavage of the chemically modified mismatches

50 μl of 1M freshly prepared piperidine solution in water was added to each pellet, tubes were mixed by vortexing for 1 minute and incubated at 90°C water bath for 30 minutes. At the end of incubation the reaction was precipitated as mentioned above. The pellets were dissolved in 10 μl of formamide loading buffer (95% deionized formamide; 10mM EDTA; 10mg/ml each of bromophenol blue and xylene cyanol).

(E) Polyacrylamide gel electrophoresis

Samples were denatured at 95°C for 5 minutes, quenched on ice and then loaded alongside a radiolabelled 1kb DNA ladder. The gel was 6% polyacrylamide containing 7M urea and prepared in 1X TBE buffer. Electrophoresis was at a constant 2000 volts for approximately 3 hours then the gel was dried and autoradiographed for 16 hours at -40°C .

2.5.12 Genomic Sequencing

The amplified PCR DNA was directly sequenced using single stranded templates. Asymmetric PCR was performed according to a protocol developed by Mgone et al (1992) in which a PCR product was used as a template to generate single stranded DNA during the PCR reaction. DNA targets to be sequenced were prepared by the standard PCR technique. Amplified products were resolved by agarose gel electrophoresis and the desired bands were cut out, placed in 1.5 ml tubes with 50 µl TE buffer and kept at -20°C overnight. 5 µl aliquots were then used for asymmetric PCR amplification using the standard protocol of 40 cycles with the concentration of either the sense or antisense primers being in 50-100X excess of the other primer. 5µl of each reaction was checked by agarose gel electrophoresis for the production of single stranded DNA. Prior to sequencing, the asymmetric PCR products were cleaned to remove excess dNTPs, salt and primers. An equal volume of 4M ammonium acetate and two volumes of isopropanol were added to each reaction, mixed briefly and incubated at room temperature for 10 minutes then centrifuged in a microcentrifuge at ~14000 rpm (12,000g) for 10 minutes. Pellets were washed in 70% ethanol, air dried and resuspended in 7 µl of ddH₂O and used directly in the sequencing reaction.

Sequencing protocol using USB sequenase version 2.0 sequencing kit

Direct sequencing was carried out by the chain termination sequencing method (Sanger et al 1977) using the Sequenase version 2.0 sequencing kit (USB). 25µl of the T7 DNA polymerase was diluted with 25 µl inorganic pyrophosphatase and 150 µl of glycerol enzyme dilution buffer to obtain an 8 fold dilution of the enzyme to its working concentration of 1.6 units/µl.

7 µl of the purified asymmetric PCR product were mixed with 2 µl 5X sequenase buffer to give a final concentration of 40 mM Tris-HCl pH 7.5, 50 mM Na Cl and 20 mM MgCl₂. 1 µl (1 pmol) of the sequencing primer, either limiting or nested and complementary to the synthesised single strand was also added. The mixture was vortexed, briefly centrifuged and annealed by heating at 65°C in a

PCR block for 2 minutes then slowly cooled to less than 35°C over 15 to 30 minutes and quenched on ice for few minutes. 1 µl of 0.1M DTT, 2 µl of a 1/5 dilution of labelling mix (1.5 µM of each dNTP except dATP), 0.5 µl of α-³⁵SdATP (1000 Ci/mmol; 10 µCi/µl) and 2 µl of a 1/8 dilution of T7 DNA polymerase were added, mixed well, briefly centrifuged and incubated at room temperature for 2-5 minutes. After the incubation, 3.5 µl of the mixture was transferred to 2.5 µl of each termination mix at 42°C incubated at 42°C for 5 minutes. The termination mixes (4 mixes) contained 80 µM of each dNTP and 8 µM of the appropriate dideoxyribonucleoside triphosphate (ddNTP). The reaction was then terminated by the addition of 4 µl formamide dye stop solution (95% formamide, 20 mM EDTA, 0.05% of each bromophenol blue and xylene cyanol). Tubes were then stored at -20°C until further need.

To read the sequencing close to the primer, 1 µl of Mn buffer (0.15M Na isocitrate, 0.1M MnCl₂) was added to the labelling mix before adding the sequenase enzyme. Samples were denatured at 75°C for 2 minutes and quenched on ice for 2 minutes. 2 µl of each termination reaction was loaded onto a prewarmed (50°C), 8% denaturing polyacrylamide gel containing 7M urea, prepared and run in 1X TTE buffer (glycerol tolerant buffer). Electrophoresis was carried out at approximately 2500 volts at 50°C for 2-4 hours depending on the area of interest. After electrophoresis the gel was dried in a vacuum gel dryer then exposed to Kodak X-Omat AR film using a cassette with intensifying screens for 16 to 40 hours at -40°C.

CHAPTER 3

RESULTS

(3) RESULTS

3.1 Cytogenetic and molecular cytogenetic studies:

3.1.1 Establishment of FISH technique and optimisation of probes:

(1) Unicolour FISH using alpha satellite DNA probes

To become familiar with the FISH technique, initial experiments were carried out with the highly repetitive sequence probes DXZ1 and PDP97 specific for the centromeric region of chromosomes X and Y respectively. These probes were used to optimise the uni-colour FISH. The DXZ1 probe was obtained commercially, pre-labelled with either biotin or digoxigenin. The probe PDP97 was available as glycerol stock in the department. This probe was grown, isolated and labelled with either biotin or digoxigenin using the standard nick translation method. The labelled probes (DXZ1, PDP97) were then hybridised to metaphase spreads from peripheral blood samples. Imaging of FISH results was carried out using the CCD system and Smartcapture programme. The concentration required to produce intense and specific signals using each of the probes was optimised and found to be 0.5-1 ng of DXZ1 and 50-100 ng of probe PDP79 per 2 cm² hybridisation area (Figure 3.1).

(A) Optimisation of X-chromosome specific probes

A total of 15 probes specific for chromosome X were used in this study (Figure 3.2). Nine were commercially available [XIST (Xq13.2), KAL (Xp22.3), DXZ4 (Xq24), whole X chromosome paint (WCPX), chromosome arm paint for the short and long arm of the X chromosome (CAP Xp/CAP Xq), telomeric sequences specific for the short and long arms of X chromosome, and DXZ1 (centromeric)] pre-labelled with either digoxigenin or biotin. The rest (6 probes) were available in the department as glycerol stocks. Three (cPT1, cPT4 cAL24) were specific for the DMD

locus at Xp21 region of the X chromosome and three (VK21, 141R, G9L) were specific for the fragile X regions (FRAXA/E) at Xq27-28. The glycerol stocks of the probes were cultured, isolated from the host cell and the extracted DNA from each probe was individually labelled with biotin and/or digoxigenin.

Initially the three DMD cosmid cPT1, cPT4, and cAL24 and the three FRAXA/E probes (VK21, 141R, G9L) were optimised individually. Different probe concentrations, competitor DNA concentrations, and hybridisation temperatures were tried and optimised to obtain clear and specific signals. The optimal final concentration of the DNA mixture (probe and competitor DNA) per 2 cm² hybridisation area was found to be 200-300 ng of the probe and 20 µg of the competitor DNA. The optimal hybridisation temperature was found to be 39-40°C for 14-16 hours incubation time (Figure 3.3). Using individual probes gave inconsistent results and occasionally weak signals. To improve the hybridisation efficiency and to obtain consistent signals, the three DMD probes were equally combined to produce a probe set (contig) at a final concentration of 200-250 ng/ 2 cm² hybridisation area. Using this probe set, consistent and clear signal was obtained (Figure 3.4a). A probe set using the three FRAXA/E probes was then similarly prepared, assessed and also found to produce satisfactory results at concentration of 250-300 ng/ 2 cm² hybridisation area (Figure 3.4b). These studies revealed the suitability of the cosmid contigs for analysis of the DMD locus at Xp21 and of FRAXA/E loci at Xq27-28.

The commercial probes used in this study (XIST, KAL, DXZ4, telomeric sequences, CAP and WCP of X chromosome) were applied following the supplier's protocols with minor modifications. Clear and strong signals were observed upon using these probes (Figure 3.5).

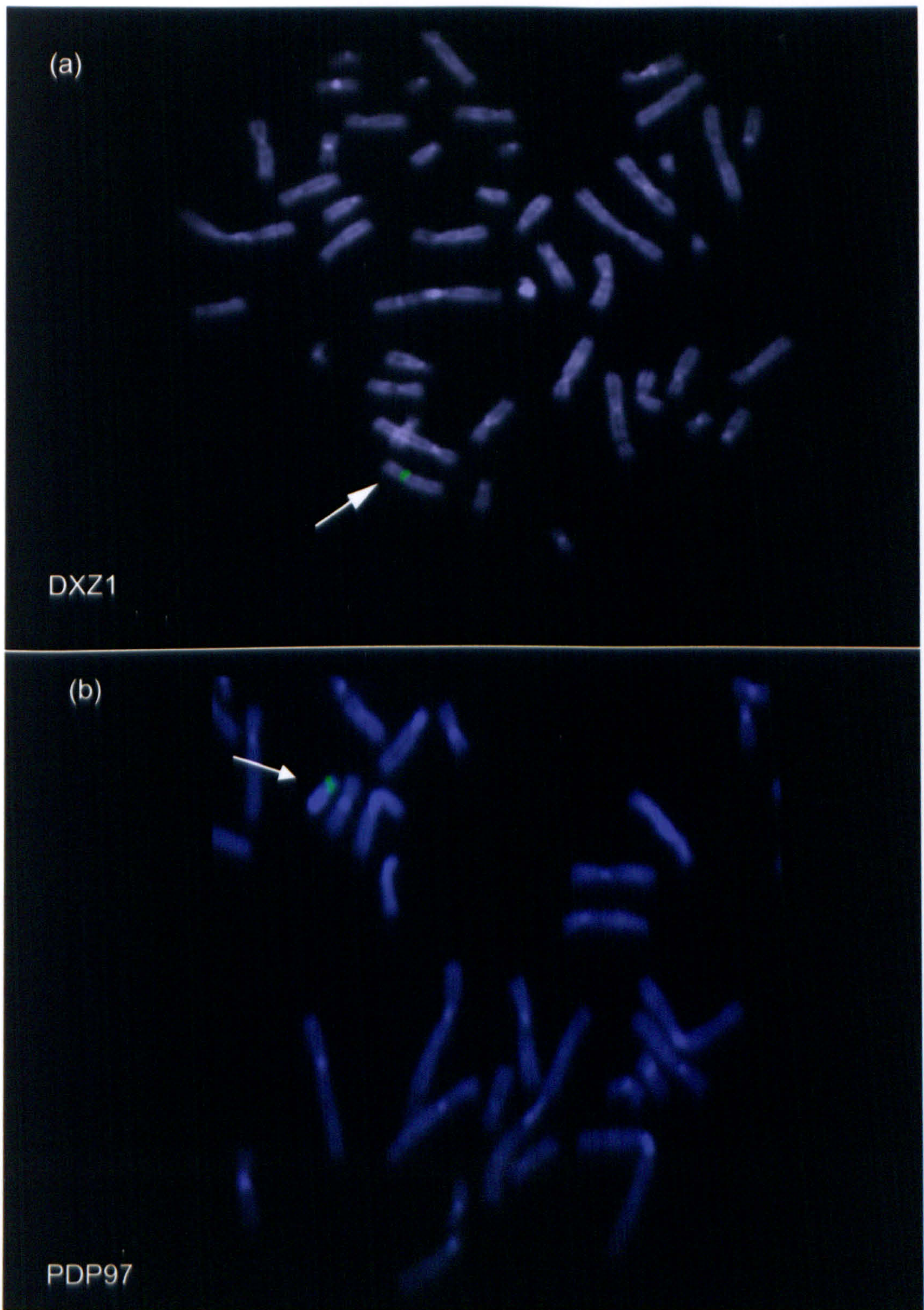


Figure 3.1: FISH analysis using biotin-labelled DXZ1 (a) and PDP97 (b) probes specific for centromeric regions of chromosomes X and Y respectively. The chromosomes were counter stained with DAPI.

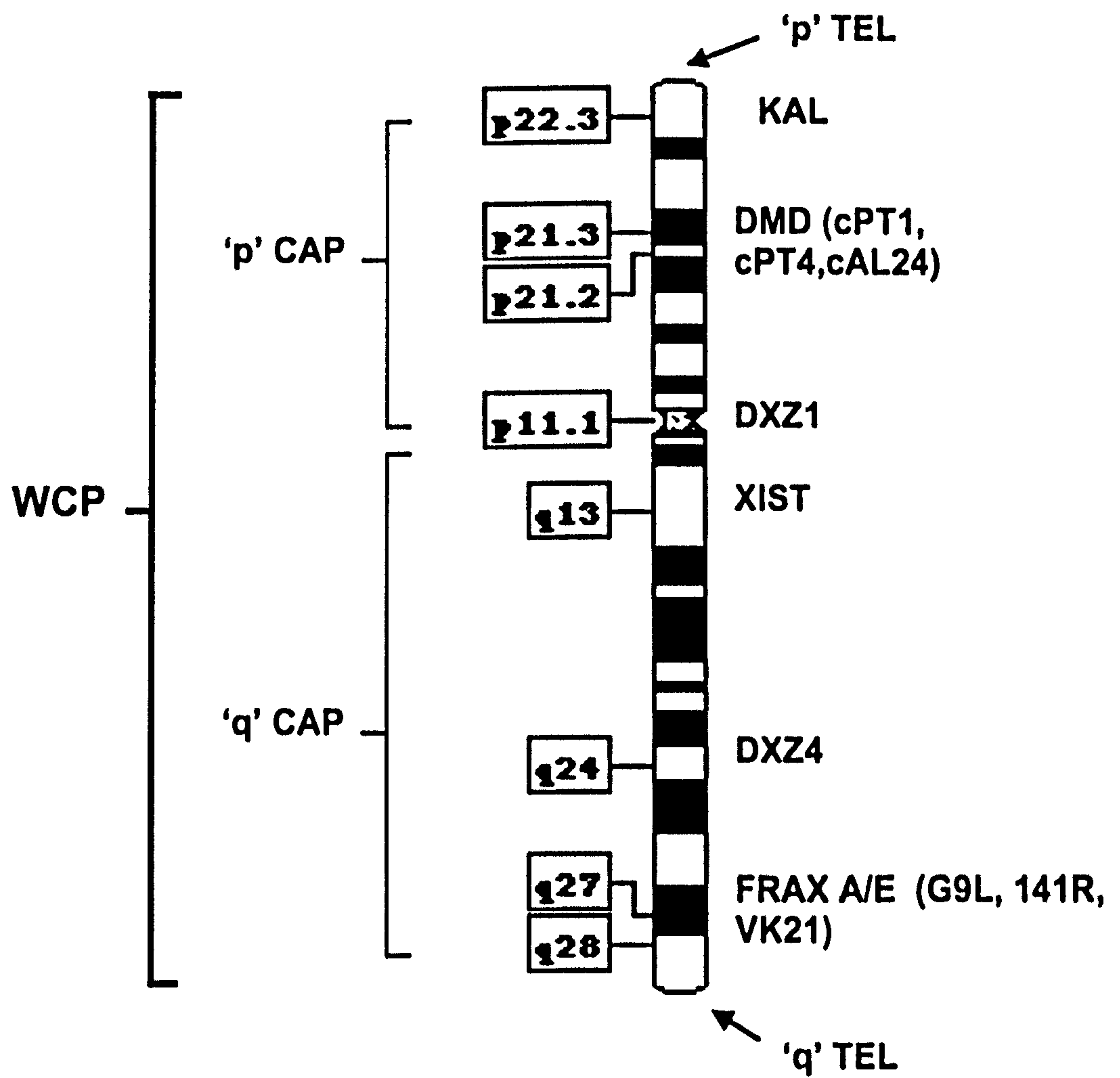


Figure 3.2: Panel of probes used for FISH study of the X chromosome

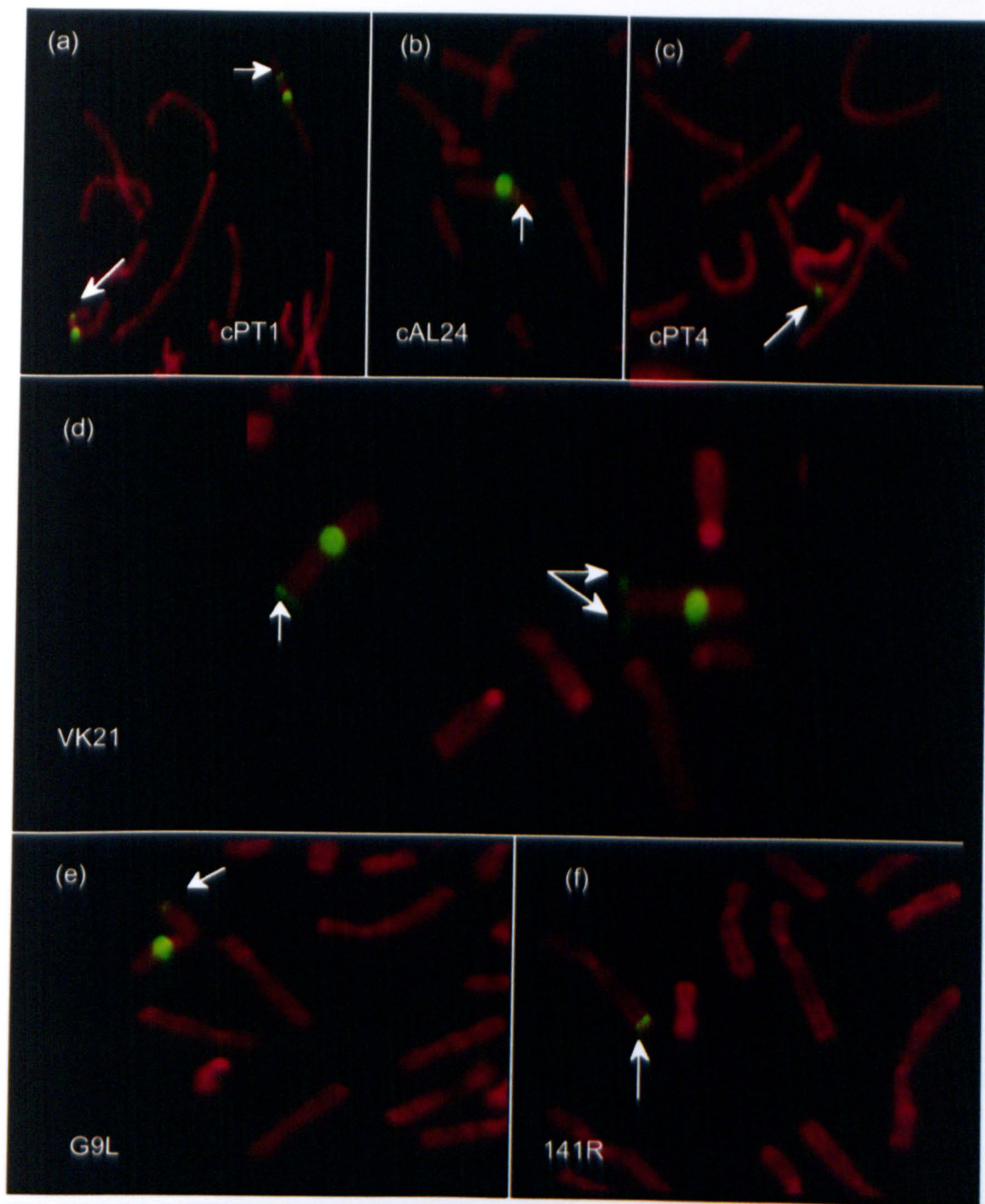


Figure 3.3: Hybridisation of cPT1, cAL24, and cPT4 probes for the DMD locus at Xp 21 region of chromosome X (a,b,c: arrowed). Figures d, e, and f represent the hybridisation of VK21, G9L, and 141R probes to the fragile site at Xp27-28 of chromosome X (arrows). The biotinylated probes were detected by fluorescein-avidin and the chromosomes were counter stained with propidium iodide.

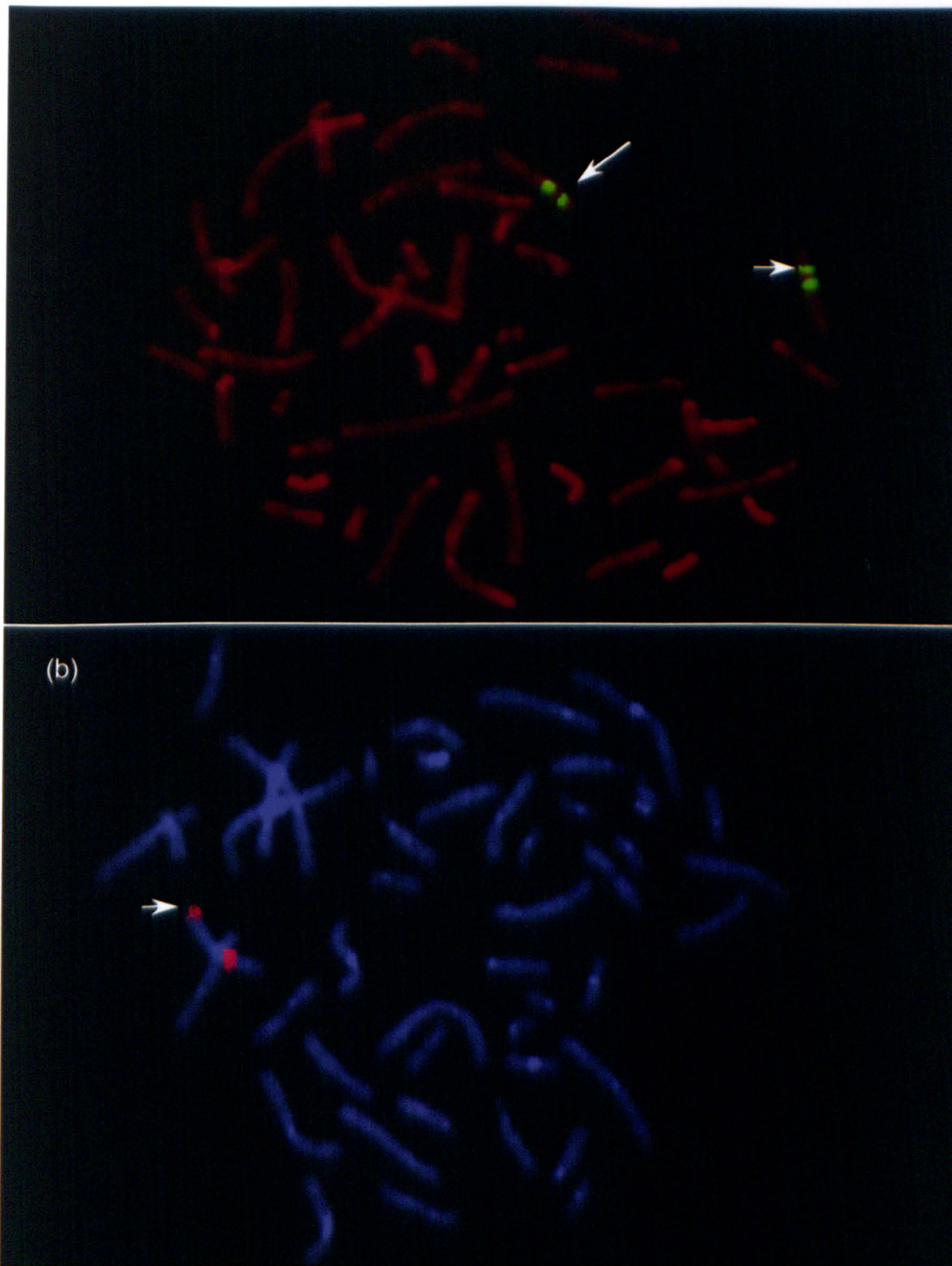


Figure 3.4: Digoxigenin- labelled DMD cosmid contig (mixture of three cosmid clones: cPT1, cPT4 and cAL24) locus probe (a) and biotin-labelled FRAXA/E cosmid contig (mixture of three cosmids: G9L, 141R and VK21) loci probe (b), both probes were co-hybridised to DXZ1 probe. FISH study showed specific and clear signals on chromosome X. The chromosomes were counterstained with propidium iodide in (a) and in (b) the chromosomes were counterstained with DAPI.

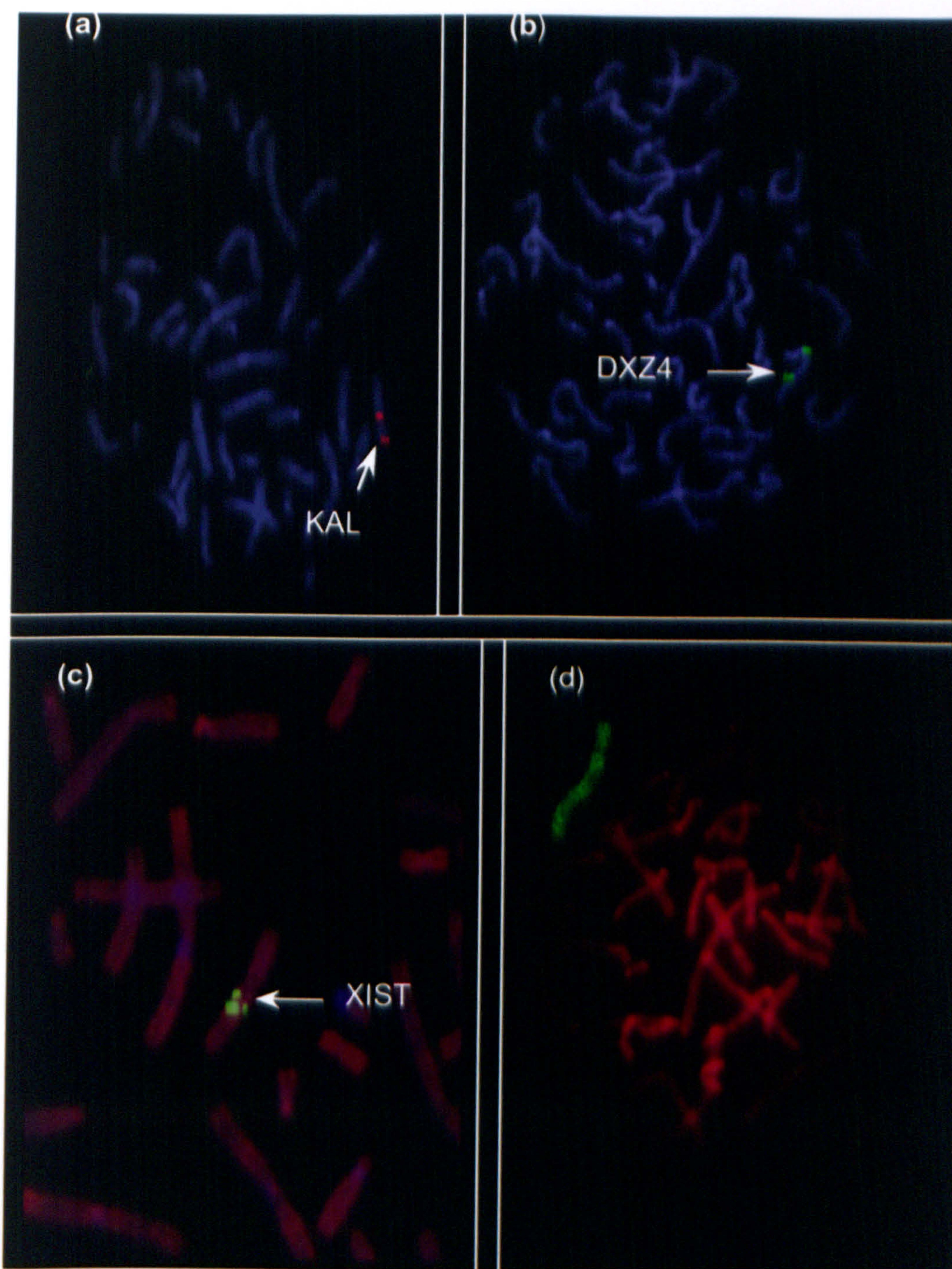


Figure 3.5: Hybridisation of KAL probe to the Kallman locus at Xp22 (a), DXZ4 probe to macrosatellite region at Xq24 (b), and the XIST probe to the X-inactivation centre at Xq13 (c) of chromosome X (arrows). Figure (d) represents the hybridisation of whole chromosome X library (WCPX) covering whole chromosome X. All locus specific probes were co-hybridised with X-centromere (DZX1) probe (a-c).

(B) Optimisation of Y-chromosome specific probes

Six different Y-specific probes (Figure 3.6) were optimised. The MK5 and MK29 cDNA probes (Ma et al, 1993) specific for azoospermia factor, and the hocSRY probe (Su and Lu 1991) specific for testicular determination factor (TDF) in male were supplied as cosmid DNA. These probes were individually transformed to an appropriate host cell for culture and preparation of glycerol stocks for further investigations. The amplified probe(s) were then isolated. Five μg of the extracted DNA was digested with the appropriate restriction enzyme and the presence of the insert was ascertained by agarose gel electrophoresis (Figure 3.7). One μg of each extracted DNA probe was labelled with either digoxigenin or biotin. Uni-colour FISH analysis was optimised using different probe concentrations, competitor DNA concentrations, hybridisation temperature, and detection method. The optimal final concentration of the DNA mixture (probe and competitor DNA)/ 2 cm^2 hybridisation area was found to be 150-200 ng of the probe and 12 μg of the competitor DNA. The optimal hybridisation temperature was between $39\text{-}40^\circ\text{C}$ for 14-16 hours incubation time (Figure 3.8). The whole chromosome Y paint (WCPY: Cambio) produced complete coverage and clear signals on the Y chromosome (Figure 3.8). The two repetitive sequence probes GMGY10 and PHY2.1 were treated exactly as the alpha satellite DNA probe without suppression with competitor DNA. The concentration of the probe(s) and the hybridisation temperature were assessed at $5\text{-}100\text{ ng}/2\text{ cm}^2$ hybridisation area and at $37\text{-}42^\circ\text{C}$ for 16 hours incubation time. For GMGY10, the concentration required to produce intense and specific signals was found to be $10\text{ ng}/2\text{ cm}^2$ hybridisation area at $39\text{-}42^\circ\text{C}$. The PHY2.1 probe was found to give strong and clear signals at a concentration of $100\text{ ng}/2\text{ cm}^2$ hybridisation area at $39\text{-}42^\circ\text{C}$ (Fig 3.8).

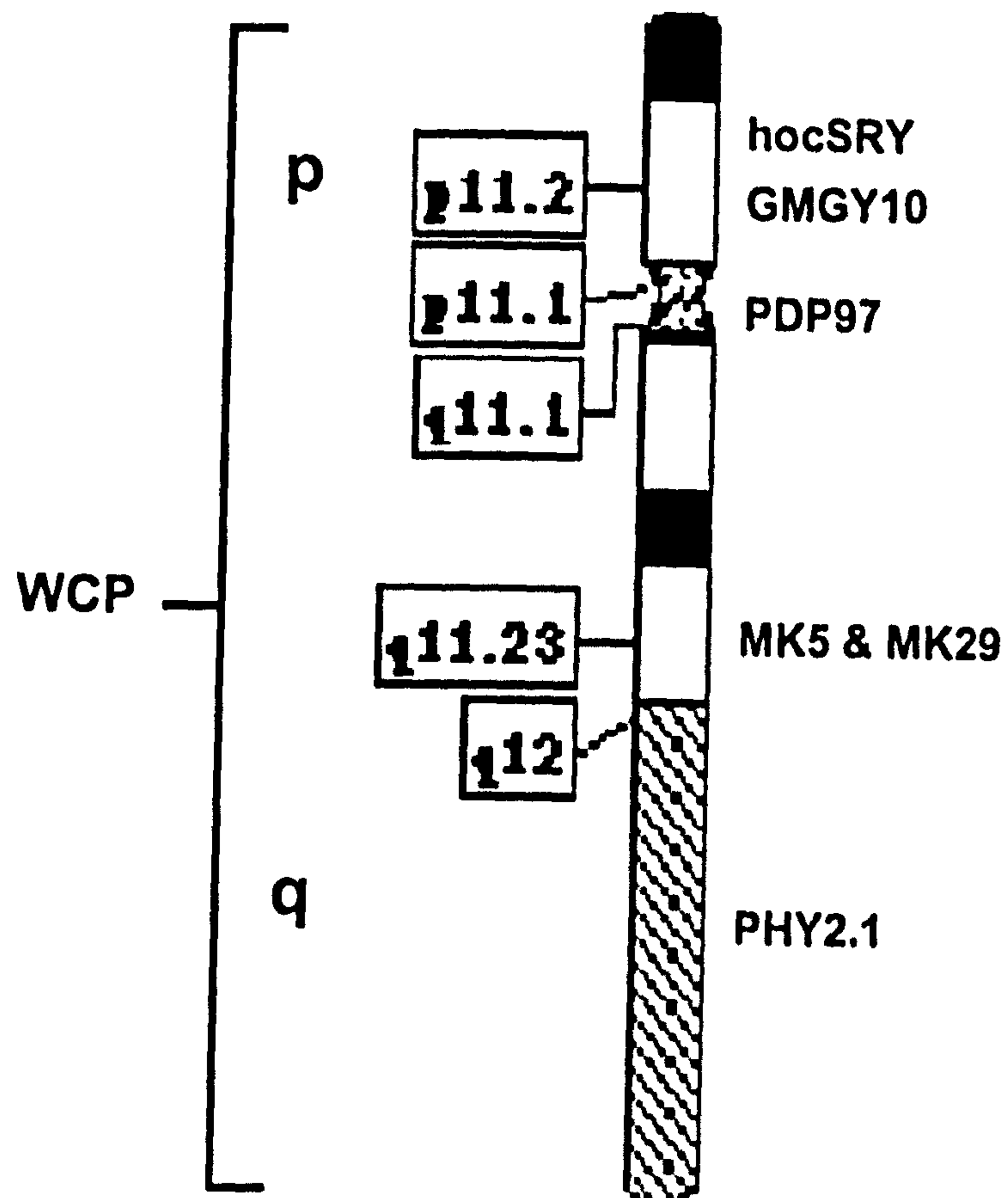


Figure 3.6:
Panel of probes used for FISH study of the Y chromosome.

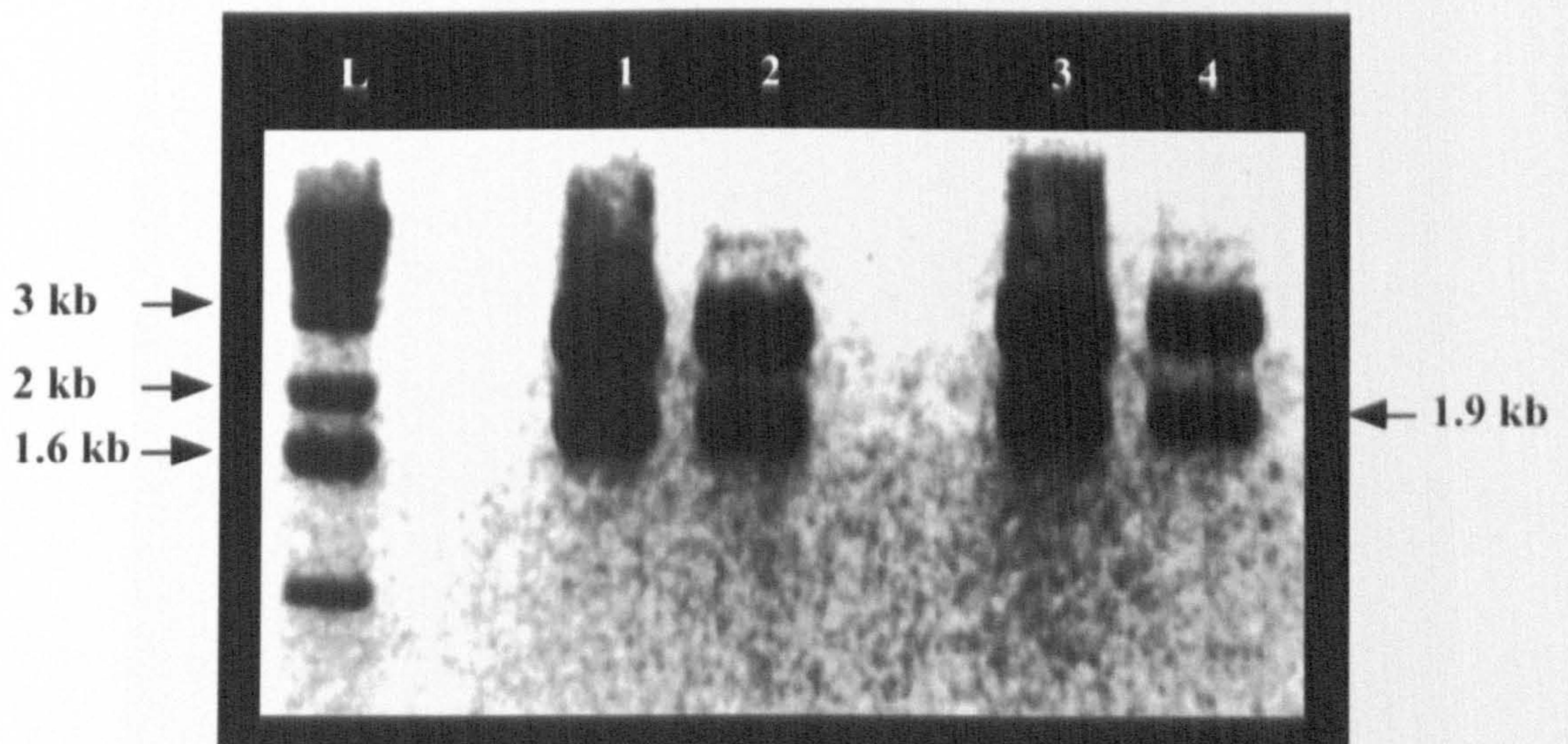


Figure 3.7: Separation of MK5 (lanes 1 and 2) and MK29 (lanes 3 and 4) cDNA inserts (1.9 kb *EcoRI* fragment) from the plasmid DNA on an ethidium bromide stained 1% agarose gel run at 10 volt/cm for one hour.
L = 1 kb ladder.

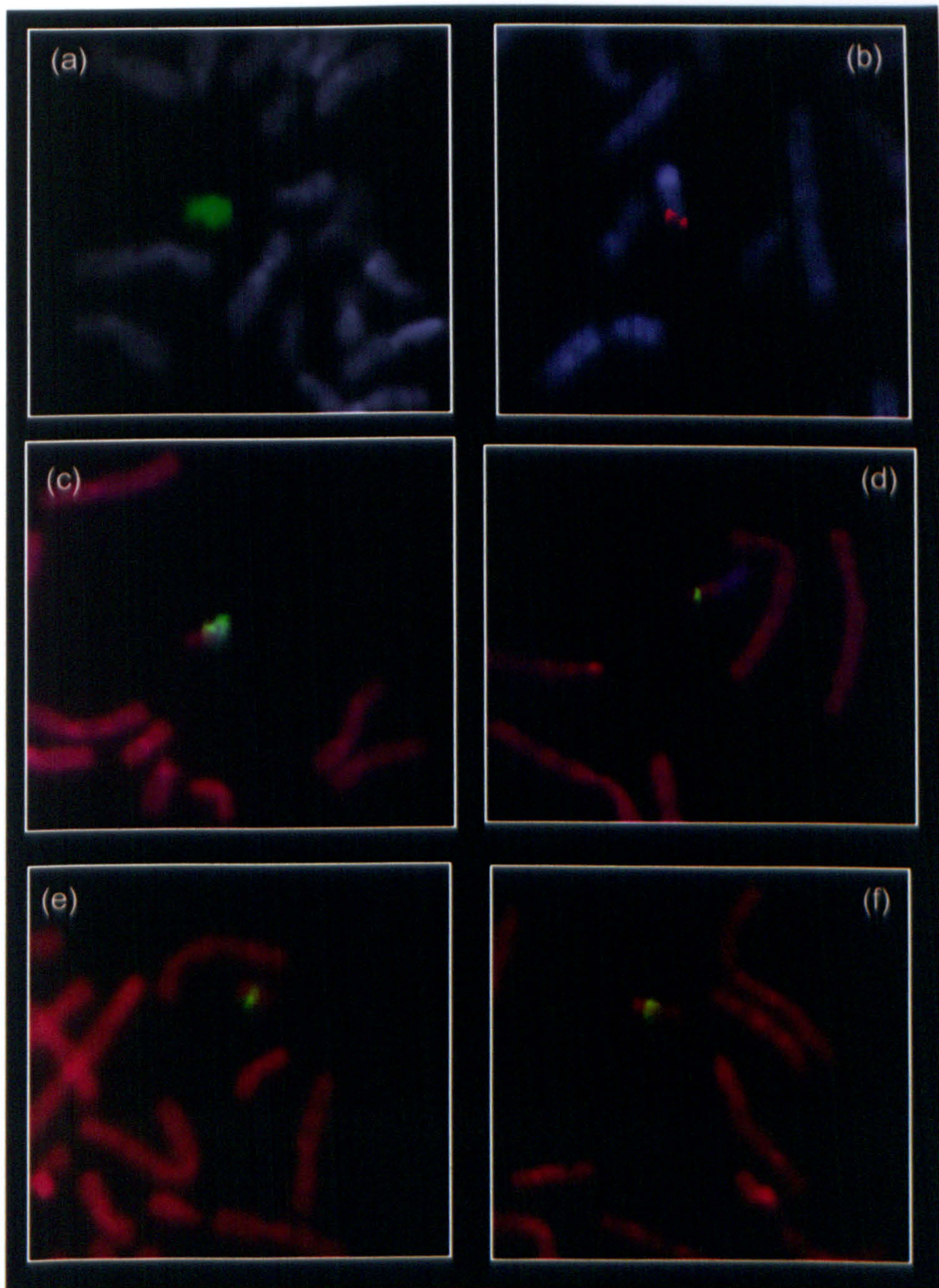


Figure 3.8: Partial metaphase spreads hybridised to different Y chromosome specific probes; (a) Y chromosome paint; (b) GMGY10 (Yp11) probe; (c) PHY2.1 (Yq12) probe; (d) SRY (Yp11.2) probe; (e and f) MK5 and MK29 cDNA probes located at Yq11. The chromosomes were counter stained with DAPI in (a,b). In figures c-f the chromosomes were detected with counter stain containing DAPI and PI.

(2) Dual and multi colour FISH

Following optimisation of uni-colour FISH, a dual FISH approach was employed to detect simultaneously two different probes hybridised to the same chromosome or to different chromosomes, in a single hybridisation experiment. A mixture of a biotin labelled probe and a digoxigenin-labelled probe was hybridised to metaphase spreads. Biotin-labelled probe was detected with rhodamine-avidin (red) and digoxigenin-labelled probe was detected with FITC (green). Each of the red and green signals was amplified using an extra layer of rhodamine-avidin and FITC. The chromosomes were counter-stained with DAPI, which allowed visualisation of both red and green signals on a blue background. The concentrations of probes used in dual FISH were guided by the optimal concentration, for each probe, used during the uni-colour FISH experiments and did not exceed a final concentration of 500 ng. Signals produced by each of the probes were strong, distinct and easily distinguished using this system. Figure (3.9a) shows a normal male metaphase spread simultaneously hybridised with digoxigenin labelled DXZ1 (green) and biotin labelled PDP97 (red) probes.

Once the reliability of the probes had been established both singly and in pairs using various detection systems, a three colour combinatorial FISH approach was carried out to detect three probes simultaneously. In this technique, one probe was digoxigenin-labelled, a second biotin-labelled and a third consisting of a 1:1 ratio of biotin and digoxigenin-labelled probe were combined in a hybridisation mixture of total volume 12-34 μ l. Detection of the probes in three colour ratio mixing FISH was performed as in the dual FISH study. Biotin labelled probes were detected with rhodamine-avidin (red) and amplified once with biotin labelled anti-avidin and rhodamine-avidin. Digoxigenin labelled probes were detected with FITC labelled monoclonal anti-digoxigenin (green) and amplified by anti-mouse conjugated with FITC. A third colour (yellow) was produced by detection of the combinatorial-

labelled probe with both rhodamine and FITC. Figure (3.9b) shows a metaphase spread simultaneously hybridised to three different probes specific for chromosome X (XIST, KAL and DXZ1). All probes used in this study were optimised to provide strong and well-defined signals on metaphase spreads. Although some slight background labelling can sometimes be observed, chromosome identification was always unambiguous.

3.1.2 Applications of FISH technique:

(1) Patients

FISH analysis was applied to two groups of patients. The first group of cases consisted mostly of Turner syndrome and its variants that had been shown cytogenetically to have a chromosome abnormality involving the sex chromosomes. The karyotypes of these patients are shown in table 3.1. The second group consisted of cases with disorders related to sex determination and sex differentiation associated with normal karyotype (Table 2.1). This group will be discussed later in the molecular part of the project.

(2) FISH studies of X and Y chromosomes aberrations

FISH studies were applied to identify more precisely the chromosomal origin of the abnormal or marker chromosome using probes specific for the X and/or the Y chromosome. The karyotypes of the investigated cases were performed by the cytogenetics laboratory of Duncan Guthrie Institute (DGI) or by the Kuwait Medical Genetic Centre (KMGC), Kuwait. Between 20-50 metaphase spreads were analysed in each case (Table 3.1). Mosaicism was detected in 17/24 cases (70.8%) of which fifteen patients (62.5%) had a mosaic karyotype with 45,X cell line in addition to the cell line of interest. Two of these had an unidentified marker of potential Y origin detected by PCR analysis performed in the DGI department (cases 19 and 23).

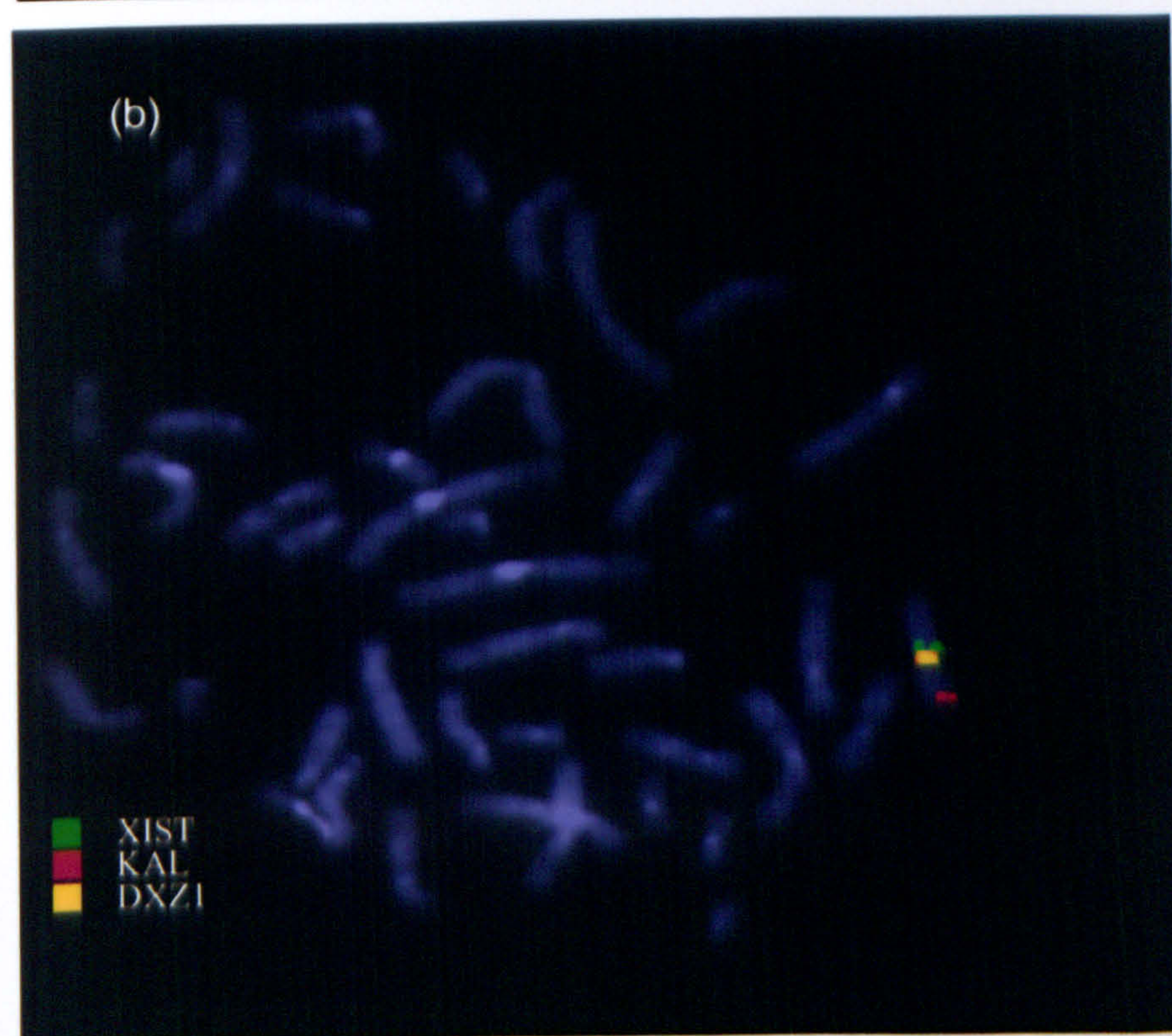
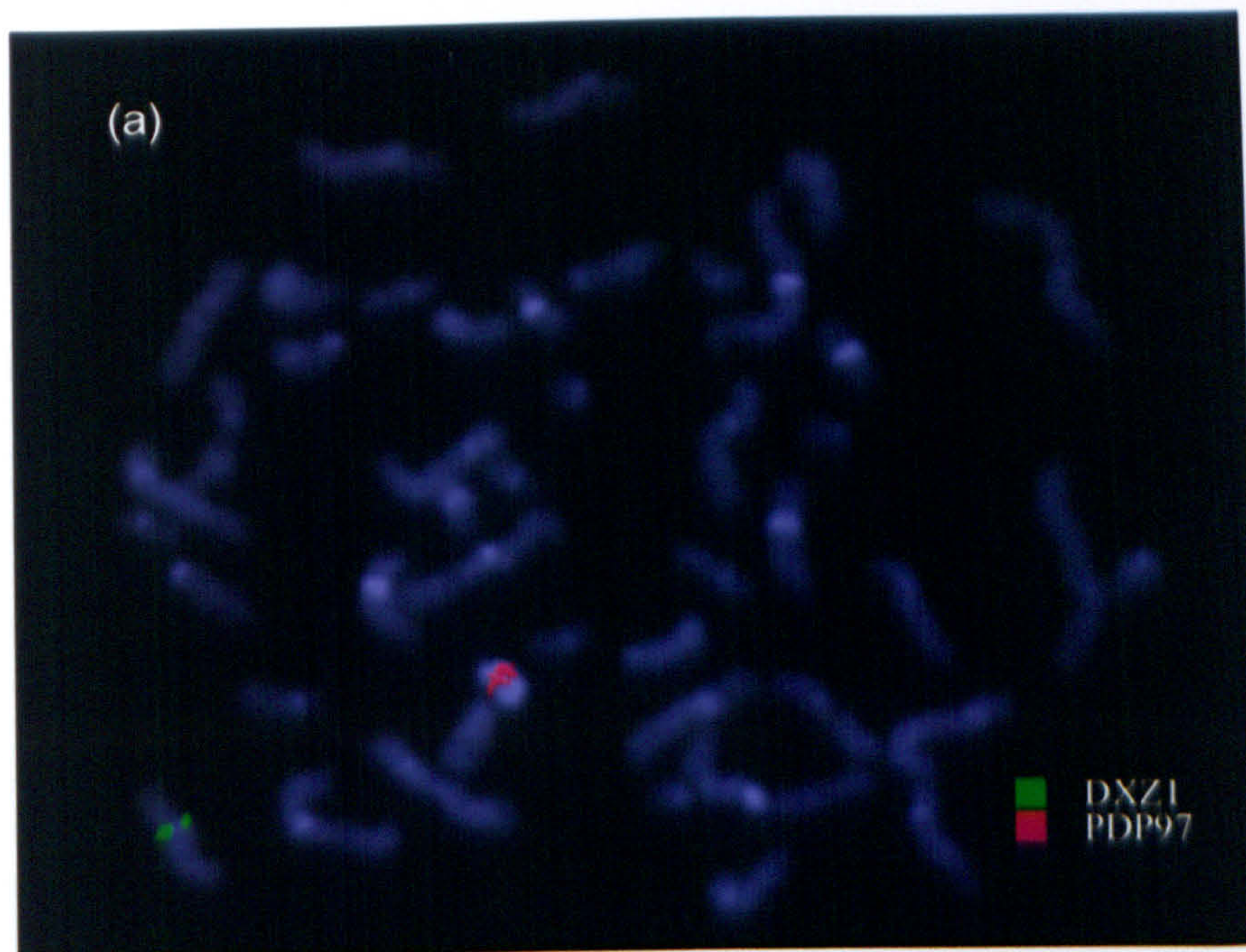


Figure 3.9: Dual colour hybridisation using digoxigenin-labelled DXZ1 and biotin-labelled PDP97 probes specific for the centromeric regions of X and Y chromosomes respectively. (b) Combinatorial FISH using digoxigenin-labelled XIST probe, biotin-labelled KAL probe and 1:1 biotin:digoxigenin-labelled DXZ1 probe. The biotin-labelled probes were detected with avidin conjugated with rhodamine. The digoxigenin-labelled detected by FITC. The chromosomes were counter stained with DAPI

Table 3.1: Details of patients with chromosomal aberrations investigated by FISH analysis.

| Case No | Cytogenetic Results | FISH Results |
|---------|--|---|
| 1 | 45,X[73]/46,X,r(X)[27] | 45,X[68]/46,X,r(X)[32]. ish (wcpX+,DXZ1+,DMD-,KAL-,XIST+,DXZ4-,wcpY-,DYZ3-) |
| 2 | 45,X[72]/46,X,r(X)[28] | 45,X[88]/46,X,r(X)[22]. ish (wcpX+,DXZ1+,DMD-,KAL-,XIST+,DXZ4-,wcpY-,DYZ3-) |
| 3 | 45,X[80]/46,X,r(X)[20] | 45,X[85]/46,X,r(X)[15]. ish (wcpX+,DXZ1+,DMD-,KAL-,XIST+,DXZ4-,wcpY-,DYZ3-) |
| 4 | 45,X[50]/46,X,r(X)[50] | 45,X[58]/46,X,r(X)[42]. ish (wcpX+,DXZ1+,KAL+,XIST+,DXZ4-,wcpY-,DYZ3-) |
| 5 | 45,X[90]/46,X,r(X)[10] | 45,X[88]/46,X,r(X)[12]. ish (wcpX+,DXZ1+,DMD-,KAL-,XIST+,DXZ4+,wcpY-,DYZ3-) |
| 6 | 45,X[78]/46,X,r(X)[22] | 45,X[71]/46,X,r(X)[19]. ish (wcpX+,DXZ1+,KAL+,XIST+,DXZ4-,wcpY-,DYZ3-) |
| 7 | 45,X[71]/46,X,r(X)[29] | 45,X[75]/46,X,r(X)[25]. ish (wcpX+,DXZ1+,KAL+,XIST+,DXZ4-,wcpY-,DYZ3-) |
| 8 | 45,X[67]/46,X,r(X)[33] | 45,X[69]/46,X,r(X)[31]. ish (wcpX+,DXZ1+,wcpY-,DYZ3-) |
| 9 | 45,X[10]/46,X,r(X)[90] | 45,X[86]/46,X,r(X)[14]. ish (wcpX+,DXZ1+,wcpY-,DYZ3-) |
| 10 | 46,X,dir dup(X)(q11.2→q22.3) | 46,X,dir dup(X)(q11.2→q22.3).ish (wcpX+,DXZ1+,DMD+,XIST++,DXZ4+) |
| 11 | 45,X[16]/46,X,inv dup(X)(q28 q21)[84] | 45,X[10]/46,X,dup(X)(q22.1→Xq28)[90]. ish (wcpX+,DXZ1+,DMD+,XIST+,DXZ4++) |
| 12 | 46,X,?i (Xp) | 46,X,i (Xp). ish (wcpX+,DMD++,FRAXA/E-) |
| 13 | 46,XX[52]/46,X,i(Xp)[48] | 46,XX[58]/46,der X,t(X;X)(p22;q22)[42]. ish (DXZ1+,DMD+,KAL++,XIST+,DXZ4-) |
| 14 | 45,X[10]/46,X,i(Xq)[83.3]/47,X,i(Xq),+i(Xq)[6.7] | 45,X[12]/46,X,i dic(Xq) [84]/47,X,i dic(Xq),+i dic(Xq)[4]. Ish(wcpX+,DXZ1++,CAP Xq++,wcpY-,DYZ3-,DYZ2-) |

Table 3.1: Continue

| <i>Case No</i> | <i>Cytogenetic Results</i> | <i>FISH Results</i> |
|----------------|----------------------------------|---|
| 15 | 46,XXp- | 46,XXp- (pter-p11). ish (wcpX+,DXZ1+,KAL-,CAP Xq+) |
| 16 | 46,XX | 46,XX |
| 17 | 45,X[40]/46,XY[60] | 45,X[42]/46,XY[58]. ish (Y) (wcpX-,DXZ1-,wcpY+,DYZ3+) |
| 18 | 45,X[52]/46,XY[48] | 45,X[48]/46,XY[52]. ish (Y)(wcpX-,DXZ1-,wcpY+,DYZ3+) |
| 19 | 45,X[94]/46,X,+mar[6] | 45,X[96]/46,X,+ mar[4]. ish (wcpX++,DXZ1-,wcpY++,DYZ3++) |
| 20 | 46,XXp+ | 46,X,-X,+ t(X;16)(p22.3;q24). ish (wcpX+,DXZ1+,KAL+,wcp16+,CAP 16q+) |
| 21 | 46,X,t(Xp;Yq) | 46,X,t(Xp;Yq). ish (DXZ1+,XIST+,DYZ3-,wcpY+,DYZ2+,MK5+,GMGY10-,SRY-) |
| 22 | 45,X[74]/46,X,i(Yq)[26] | 45,X[67]/46,X,i(Yq)[33]. ish (wcpX-,wcpY+,GMGY10-,SRY-,DYZ2++) |
| 23 | 46,XY[88]/46,X,+ mar (?derY)[12] | 46,XY[91]/46,X,i(Yq) [6]. ish (MK5++,wcpY+,DYZ3+) /46,X,r(Y)[3].ish (wcpY+,DYZ3+,GMGY10+,DYZ2+) |
| 24 | 48,XXXX | 48,XXXX |

Structural abnormalities of X or Y were confirmed in 20/ 24 (83.3%) cases, including ring X, ring Y, i(Yq), isochromosome of the short arm and long arm of X chromosome, deletion of Xp, duplication of Xq, as well as X-autosome and X-Y translocations. Seventeen of the investigated cases had X chromosome abnormalities (70.8%%) and six had Y chromosome aberrations (25%). Table 3.1 summarises all the findings obtained by FISH studies.

The objective in studying these cases was to obtain, if possible, additional information about the composition of the abnormal chromosome, to confirm the origin of the material present and to identify breakpoints more precisely.

(A) FISH studies of cases with X chromosome abnormalities

(1) FISH results from 9 Turner syndrome cases with ring chromosome

The subjects of this study were females ascertained because of multiple congenital abnormalities or with features of Turner syndrome, with or without learning difficulties (cases 1-9). The clinical and cytogenetic findings of these patients are summarised in table 3.2. Routine cytogenetic analysis of 20 metaphases showed predominantly 45,X cell line and a second cell line containing a ring chromosome. Therefore a total of 100 cells were scored in all of the mosaic cases. In four of these cases the ring was small (smaller than chromosomes 21 and 22). Further identification was not possible by conventional cytogenetic techniques on any of these. Four cases contained a medium sized ring (size of chromosomes 16,17 and 18) and were thought to contain material from the X chromosome.

In the remaining case (Table 3.2, L.D) the ring was large but contained only one centromere, and had been identified in the routine laboratory as containing both long and short arm regions of the X chromosome. However given the lack of banding in the ring chromosomes, content and breakpoints could not be unequivocally

identified and FISH studies were undertaken to identify the content of these ring chromosomes.

(a) Detection of the origin of the ring chromosomes by FISH

Initially slides from all nine cases were hybridised with whole chromosome libraries for the X and Y chromosomes and with centromeric probes DXZ1 (X) and PDP97 (Y). All the ring chromosomes showed complete coverage with the X chromosome library, and the X centromeric probe showed one signal in each ring (Figure 3.10). No signals were obtained in hybridisation experiments with the two Y probes, and it was concluded that each ring was derived from X chromosome material. FISH analysis using DXZ1 in 100 randomly selected metaphases confirmed the presence of the two cell lines (45,X/46,X,r(X)) with the percentage shown in the inserted table. The rings varied in size from small to large ring X (Table 3.2). The frequency of the second cell line r(X) varied from 12% to 42%.

| <i>Case No</i> | <i>Cytogenetic results</i> | <i>FISH results</i> |
|----------------|----------------------------|------------------------|
| 1 | 45,X[73]/46,X,r(X)[27] | 45,X[68]/46,X,r(X)[32] |
| 2 | 45,X[72]/46,X,r(X)[28] | 45,X[78]/46,X,r(X)[22] |
| 3 | 45,X[80]/46,X,r(X)[20] | 45,X[85]/46,X,r(X)[15] |
| 4 | 45,X[50]/46,X,r(X)[50] | 45,X[58]/46,X,r(X)[42] |
| 5 | 45,X[90]/46,X,r(X)[10] | 45,X[88]/46,X,r(X)[12] |
| 6 | 45,X[78]/46,X,r(X)[22] | 45,X[71]/46,X,r(X)[19] |
| 7 | 45,X[71]/46,X,r(X)[29] | 45,X[75]/46,X,r(X)[25] |
| 8 | 45,X[67]/46,X,r(X)[33] | 45,X[69]/46,X,r(X)[31] |
| 9 | 45,X[90]/46,X,r(X)[10] | 45,X[86]/46,X,r(X)[14] |

(b) Analysis of r (X) s breakpoints by FISH

The ring chromosomes (cases 1-7) were hybridised to the panel of X chromosome probes and the results are shown in Table 3.3. As can be seen, it was possible to differentiate one of the medium sized rings which contained more long arm material [(case 7) (Figure 3.11)] from the other three which contained more short arm material [(cases 4, 6 and 7) (Figure 3.12-14)]. FISH analyses showed that the breakpoints could be distal to Xp21 and Xq24 in case 5 (C.B) and could be distal to Xp22 and Xq13 in the other three cases with medium size ring X (cases 4, 6 and 7). FISH study also showed that the cases with medium size ring (cases 3-7) also contained the locus for X-inactivation (XIST) (Table 3.3 and Figures 3.11-3.14).

It was only possible to study three of the four small rings by FISH. All three were shown to contain XIST locus (Xq13) and the more distal probes for both the long and short arm showed no signal (Figure 3.15-17). Cases 8 and 9 could not be studied in detail due to lack of materials. Unfortunately, time did not permit the use of additional probes, which might improve the resolution of this study on ring chromosomes.

Table 3.2: Clinical and cytogenetic findings in nine patients with a ring X chromosome cell line.

| ID | (1) W.H | (2) D.C | *(3) J.H | (4) F.R | (5) C.B | (6) C.F | (7) K.R | (8) L.D | (9) H.A |
|-----------------------------|---------|---------|----------|---------|---------|---------|---------|---------|---------|
| Short stature | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Gonadal dysgenesis | Yes | Yes | Yes | S.P. | S.P. | Yes | Yes | S.P. | Yes |
| Widely spaced nipples | Yes | Yes | Yes | - | No | No | No | No | Yes |
| Webbed neck | No | No | No | No | No | No | No | No | No |
| Short neck | No | No | Yes | No | No | No | No | Yes | No |
| Pectus excavatum | No | No | No | No | No | No | No | No | No |
| Congenital lymphoedema | No | No | Yes | No | No | No | No | No | No |
| Epicanthic folds | No | No | No | - | No | - | - | No | No |
| Low posterior hair line | No | No | Yes | Yes | No | No | No | Yes | - |
| Narrow hyperconvex nails | Yes | Yes | Yes | Yes | No | No | Yes | Yes | - |
| Cubitus valgus | Yes | Yes | Yes | Yes | No | Yes | Yes | Yes | - |
| Short 4th metacarpal | No | No | No | No | No | No | No | No | - |
| Narrow palate | Yes | Yes | Yes | No | No | No | No | No | - |
| Small jaw | No | No | No | No | No | No | No | No | - |
| Excess pigmented naevi | No | No | Yes | No | No | No | No | Yes | - |

Table 3.2: ‘Continue’

| ID | (1) W.H | (2) D.C | ** (3) J.H | (4) F.R | (5) C.B | (6) C.F | (7) K.R | (8) L.D | (9) H.A |
|-----------------------|---------|------------------|------------------|---------|---------|------------------|---------|---------|---------|
| Glue ear hearing loss | Yes | Yes | Yes | No | No | Yes | Yes | Yes | - |
| Squint | - | Yes | Yes | No | No | Yes | No | No | - |
| Cardiac anomaly | No | No | No | No | No | No | No | No | - |
| Renal anomaly | No | No | No | No | No | No | No | - | - |
| General IQ | Nr | Nr | Nr | Nr | N | Nr | Nr | - | Nr |
| | | Needs extra help | Needs extra help | | | Needs extra help | | | |
| Parental error | N/D | Pat | Mat | Pat | - | Mat | N/D | Pat | N/D |
| % r(X) cells | 27 | 28 | 20 | 50 | 10 | 22 | 29 | 33 | 10 |
| Size of r(X) | SM | SM | SM | MED | MED | MED | MED | LRG | SM |
| % Replication of r(X) | | | | | | | | | |
| Late | 50 | 50 | 46 | 86 | 88 | 69 | 80 | N/D | N/D |
| Early | 50 | 50 | 54 | 14 | 12 | 31 | 20 | N/D | N/D |

Nr. = Normal; SM = Small; MED = Medium; LRG = Large; Pat = Paternal; Mat = Maternal;
N/D = Not Done; S.P. = spontaneous Puberty. ** This patient has also autoimmune thyroiditis.

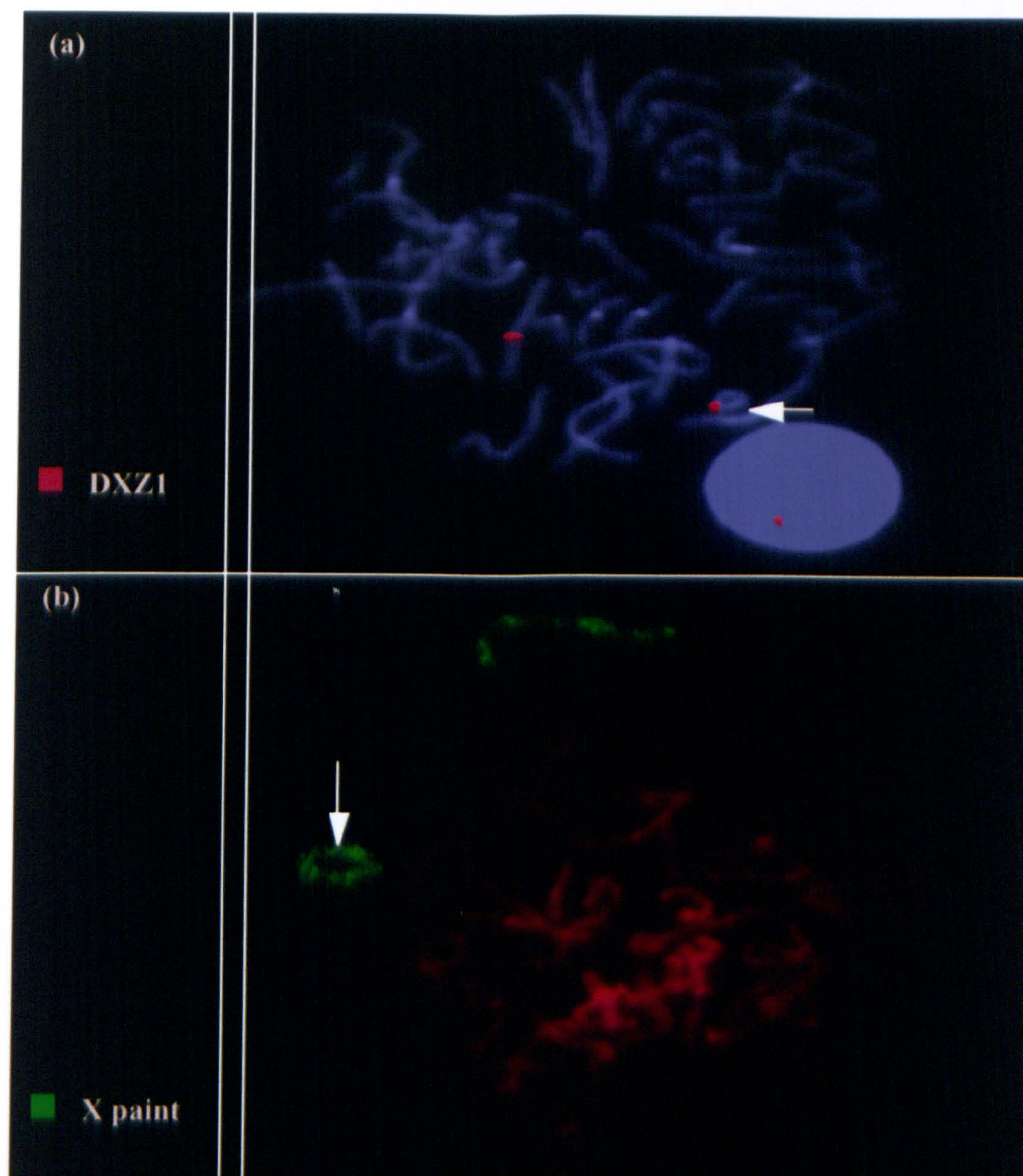


Figure 3.10: Examples of FISH analysis of ring X chromosomes using DXZ1 (a) and whole chromosome library of the X (X paint) (b). Arrows indicate the ring X chromosome. The chromosomes were counter stained with DAPI in (a) and with PI in (b).

Table 3.3: Breakpoint analysis of ring X chromosome by FISH.

| I.D. | Ring Size | WCPX | DXZ1 | DMD (Xp21) | KAL (Xp22) | XIST (Xq13) | DXZ4 (Xq24) | WCPY | PDP97 (DYZ3) |
|--------|-----------|------|------|------------|------------|-------------|-------------|------|-----------------|
| 1-W.H | Small | +ve | +ve | -ve | -ve | +ve | -ve | -ve | -ve |
| 2-D.C | Small | +ve | +ve | -ve | -ve | +ve | -ve | -ve | -ve |
| 3-J.H | Small | +ve | +ve | -ve | -ve | +ve | -ve | -ve | -ve |
| 4-F.R | Medium | +ve | +ve | ND | +ve | +ve | -ve | -ve | -ve |
| 5-*C.B | Medium | +ve | +ve | -ve | -ve | +ve | +ve | -ve | -ve |
| 6-C.F | Medium | +ve | +ve | ND | +ve | +ve | -ve | -ve | -ve |
| 7-K.R | Medium | +ve | +ve | ND | +ve | +ve | -ve | -ve | -ve |
| 8-L.D | Large | +ve | +ve | ND | ND | ND | ND | -ve | -ve |
| 9-H.A | Small | +ve | +ve | ND | ND | ND | ND | -ve | -ve |

* This patient carries a medium size ring X containing long arm materials. ND = Not done

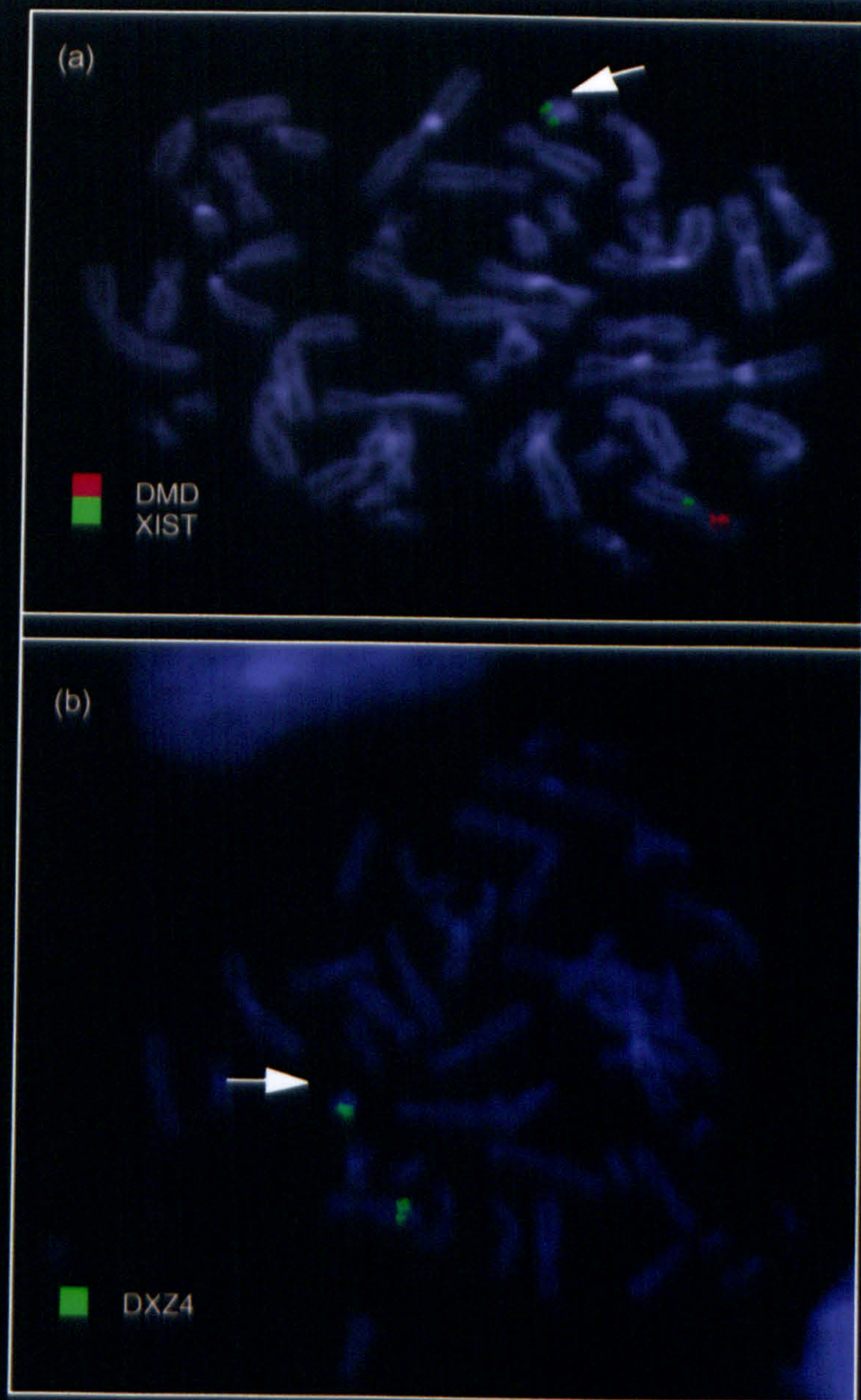


Figure 3.11: FISH analysis using three different X specific probes (DMD, XIST and DXZ4) on metaphase chromosomes from a Turner patient (C.B) with a medium size ring. The hybridisation study revealed the presence of XIST (Xq13) and DXZ4 (Xq24) on the ring X chromosome while the DMD locus was absent from the ring X chromosome. These results indicated that this case contained more long arm material. Arrow = ring chromosome.

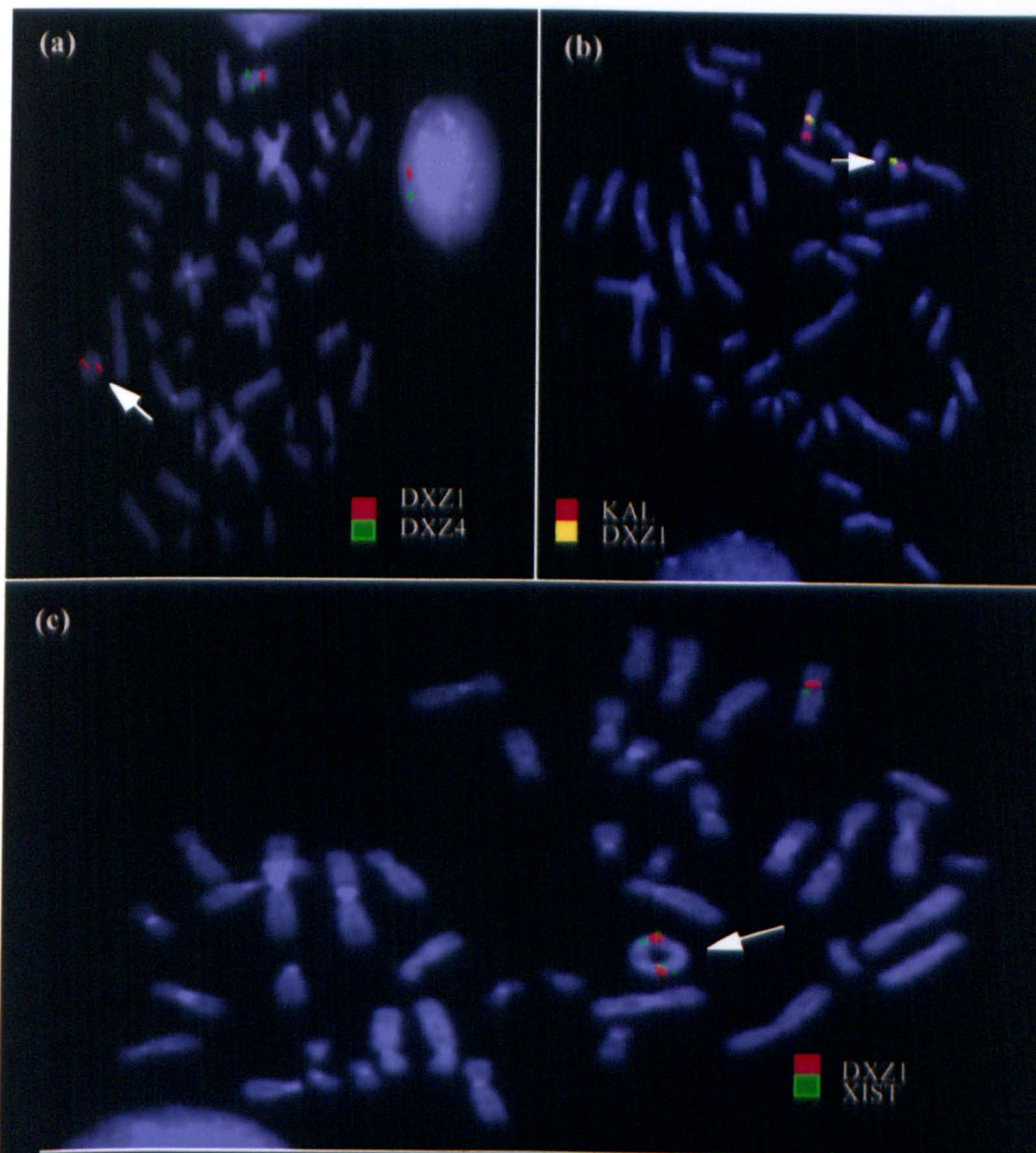


Figure 3.12: FISH study on metaphase chromosomes from patient F.R. with a medium size ring X chromosome. Hybridisation signals were detected on the r(X) for DXZ1, KAL, and XIST loci (b,c). 2% of the r(X) were observed to have two centromeres and XIST loci (c). No hybridisation signals for DXZ4 (Xq24) were observed on the r(X), although clear signals for DXZ4 were observed on the normal X chromosome (a). Arrow = ring X chromosome.

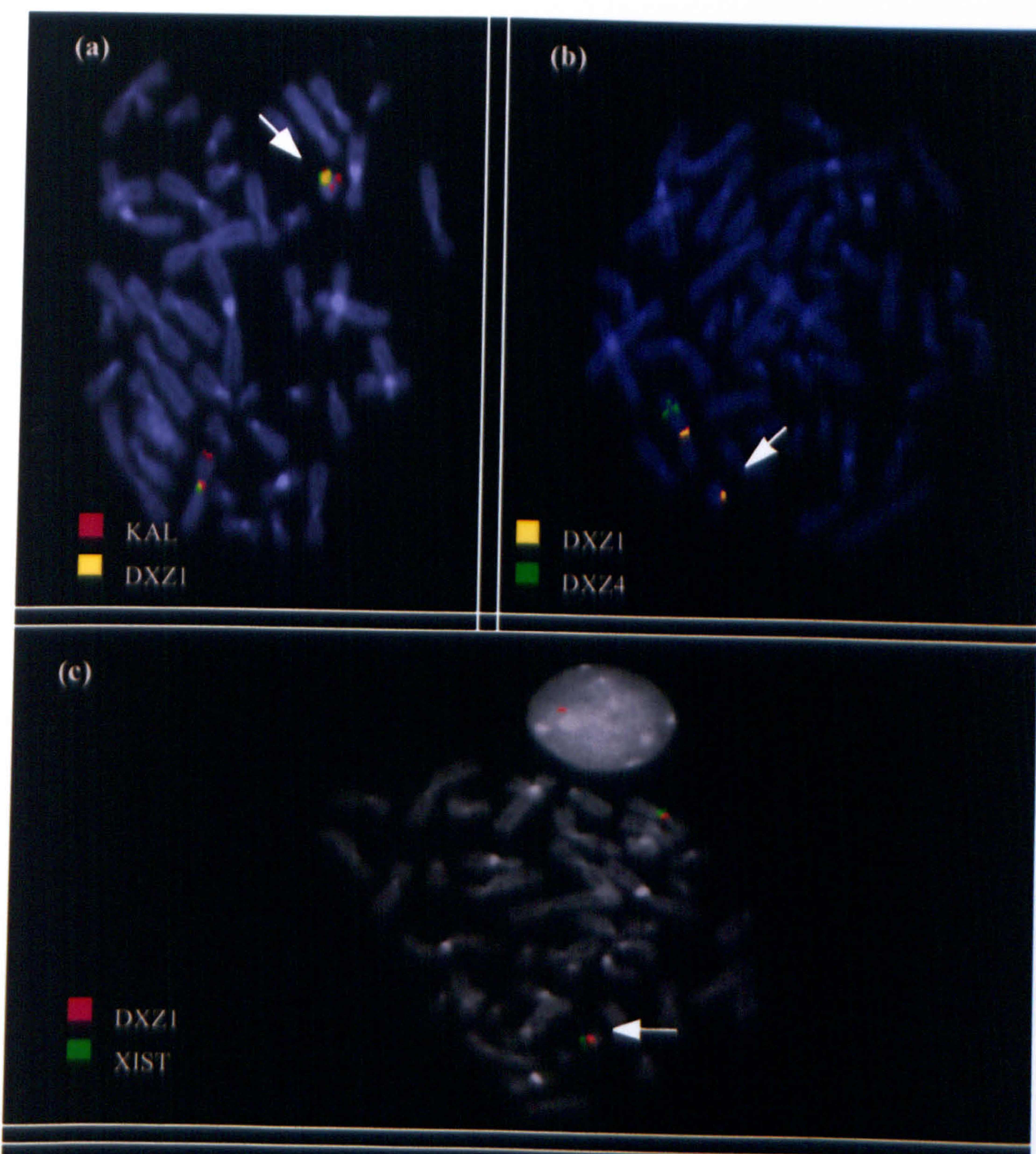


Figure 3.13: *In situ* hybridisation of X chromosome-specific probes (DXZ1, KAL, DXZ4 and XIST) to chromosomes from a patient (K.R.) with a medium size ring. Hybridisation signals of DXZ1, KAL, and XIST loci were detected on the r(X) chromosome (a, c). No signals for the DXZ4 probe were detected on the r(X) (b), although clear signals were observed on the normal X chromosome. Arrow = ring X chromosome [r(X)]. A combinatorial FISH using 1:1 biotin :digoxigenin-labelled DXZ1 probe (yellow) was used in (a,b). Chromosomes were counter stained with DAPI.

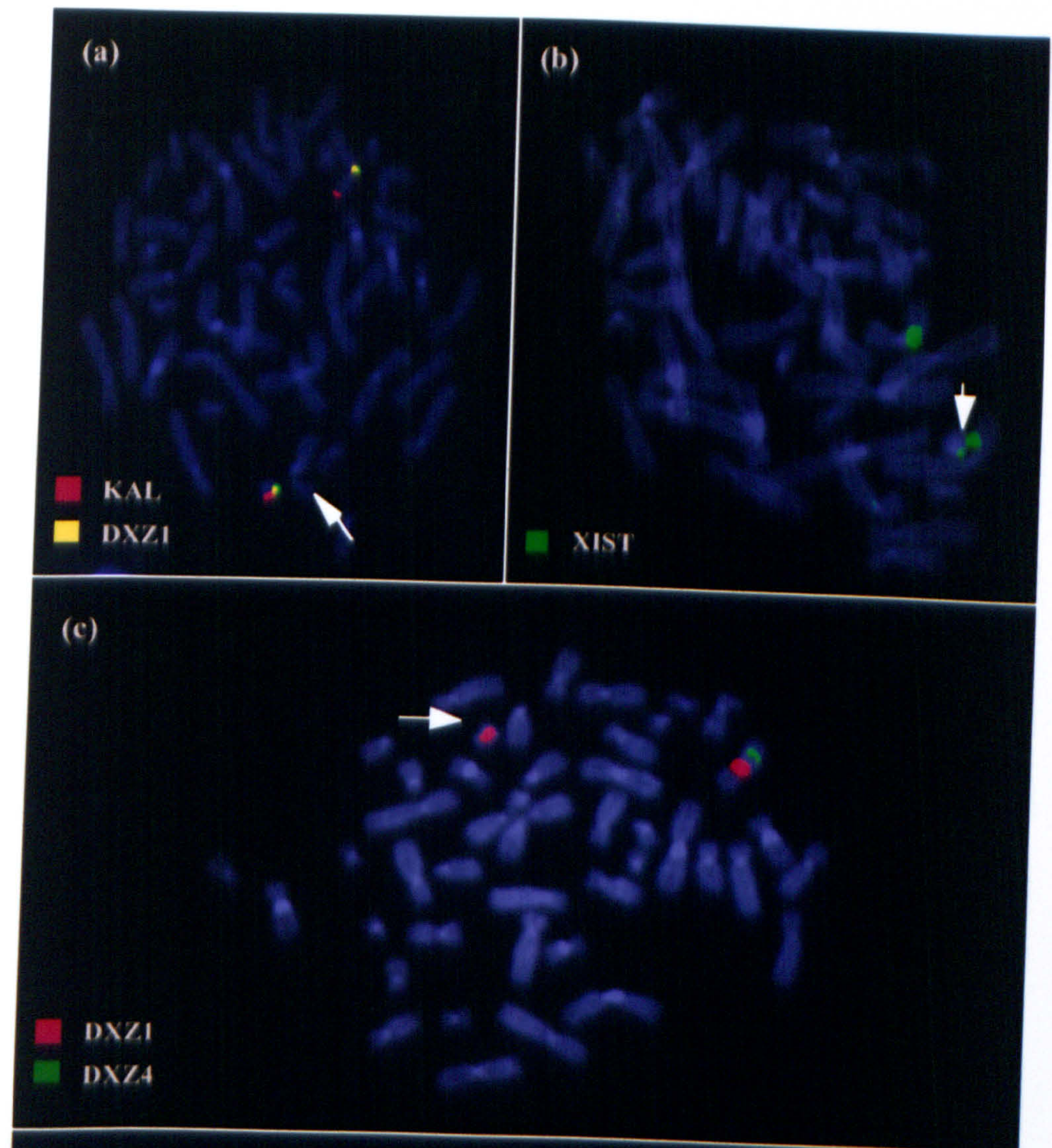


Figure 3.14: FISH analysis of a Turner patient (C,F) with a medium size ring (X). (a) A combinatorial FISH using 1:1 biotin:digoxigenin-labelled DXZ1 probe (yellow) and biotin-labelled KAL (red) showed signals on both the normal and on the ring X chromosomes. (b) Application of XIST probe showed hybridisation signals on both normal and r(X) chromosomes. (c) Dual FISH using DXZ1 and DXZ4 probes revealed that all examined rings were negative for the DXZ4 locus. Arrow = ring X chromosome.

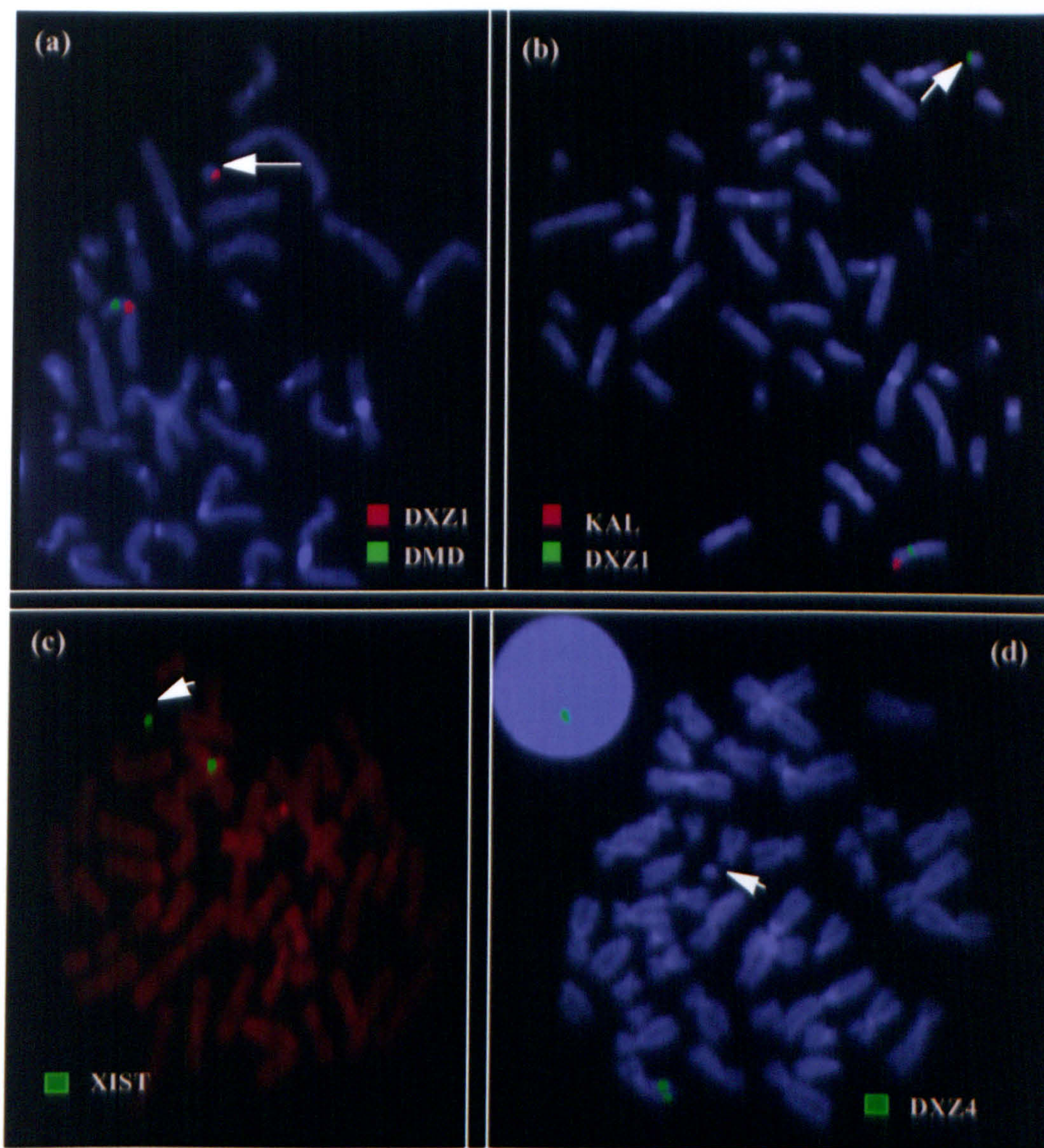


Figure 3.15: FISH study on metaphase chromosomes from patient J.H. with a small ring X chromosome. Hybridisation signals were detected on the small ring X chromosome for both DXZ1 and XIST probes (a, c). No hybridisation signals for DMD, KAL and DXZ4 probes were detected on the small ring chromosome (a, b, d; arrows). Clear hybridisation signals of all probes were seen on the normal X chromosome, DXZ1 = centromeric probe; DMD = Xp21; XIST = Xq13; KAL = Xp22.2; DXZ4 = Xq24.

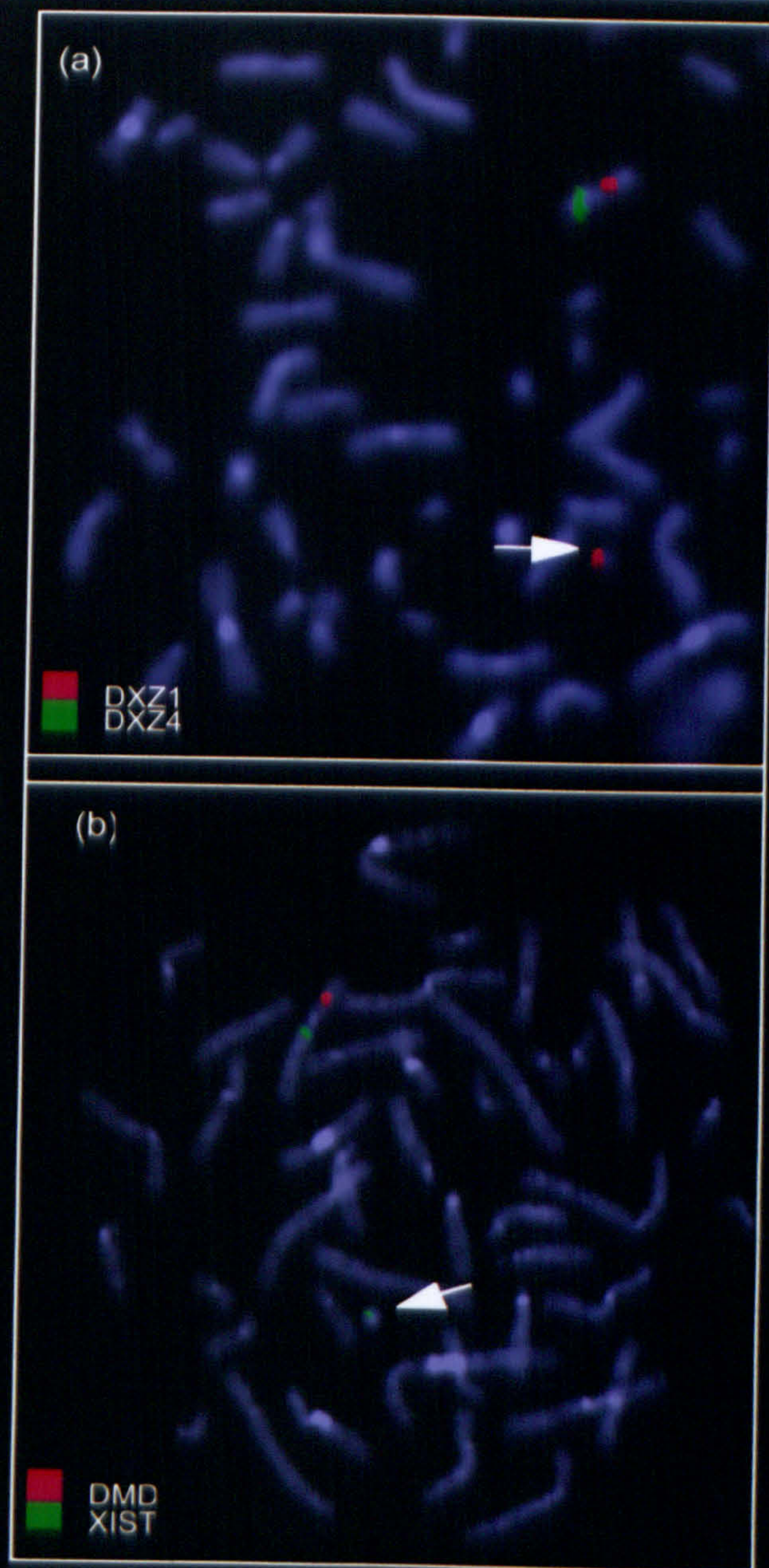


Figure 3. 16: *In situ* hybridisation signals of DXZ1 and XIST loci were observed over the normal and the ring X chromosomes (a, b) from the Turner patient (W.H.). The DXZ4 (Xq24) and the DMD (Xp21.2) loci were absent from the r(X) while clear signals were detected on the normal X chromosome (a, b). Arrow = r(X).

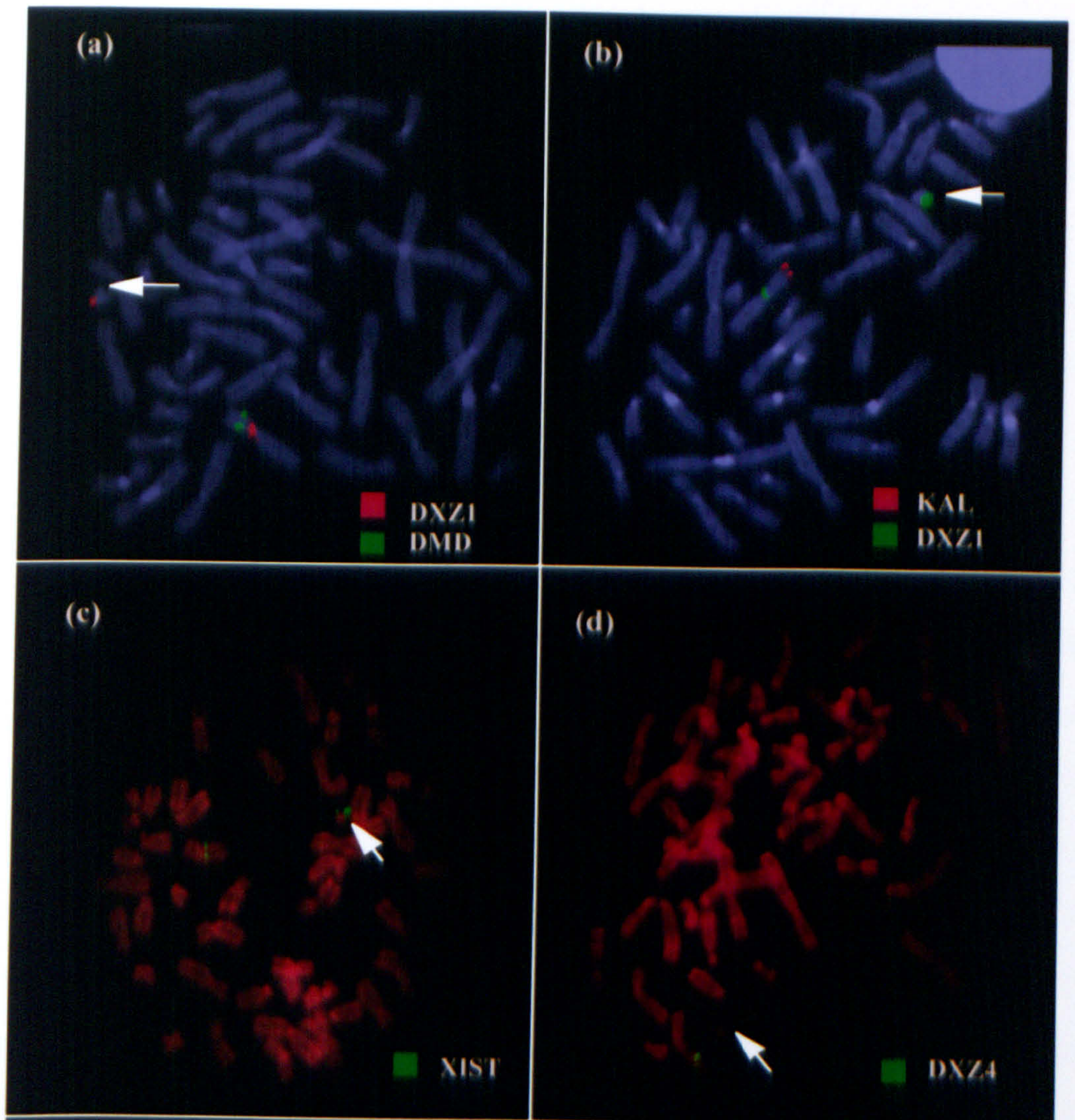


Figure 3.17: *In situ* hybridisation of metaphase chromosomes from patient D.C. with probes DXZ1, DMD, KAL, XIST and DXZ4. Hybridisation was detected on the small ring X chromosome for both DXZ1 and XIST probes (a,c). No hybridisation signals for DMD, KAL, and DXZ4 were detected on the ring X chromosome (a, b, d), although clear signals of all used probes were seen on the normal X chromosome. DXZ1 = centromeric probe; DMD = DMD locus at Xp21; XIST = XIST locus at Xq13; KAL = Kallman locus at Xp22.2; DXZ4 = macrosatellite marker at Xq24.

(2) FISH analysis of duplication Xq

Two cases with some Turner-like features (Cases 10 and 11) were referred for FISH investigation to identify and characterise their abnormal X chromosome. Routine cytogenetic analysis with G-banding in case 10 showed 46,X,dir dup(X)(q11.2→q22.3) karyotype, and case 11 had mos 45,X/46,X,dup(X)(q28→q21.1) karyotype suggesting an inverted duplication.

Case 10: A female (D.O.B 2.7.82) referred to DGI with clinical features of febrile seizure, poor growth and deafness (right ear). She had short stature and some mild stigmata of TS. Routine cytogenetic analysis of G-banding confirmed, 46,X,dir dup(X)(q11.2→q22.3) karyotype.

Initially slides from this case were hybridised with whole chromosome library for the X chromosome in order to determine if the extra material of the abnormal X was derived from X chromosome or another chromosome. FISH study using whole chromosome library for X chromosome on metaphase spreads showed a complete coverage of the normal and abnormal X chromosomes and revealed the extra chromosomal region to be either duplication of Xq or other X chromosome material (Figure 3.18a). Slides were then hybridised to a panel of X chromosome specific probes, DXZ1 (centromere), DMD (Xp21), XIST (Xq13) and DXZ4 (Xq24). FISH study of this case showed that the patient had two copies of XIST locus (Xq13) and one copy of DXZ4 (Xq24) on the long arm of the abnormal X chromosome and one copy of the DMD locus on the short arm of both X chromosomes (Figure 3.18b). These results indicated an interstitial duplication of Xq region confirming and refining the cytogenetic interpretation with the final karyotype being 46,X,dir dup(X)(q11.2→q22.3).

| Case # 10 | WCPX | DXZ1 X-cent | DMD Xp21 | XIST Xq13 | DXZ4 Xq24 |
|------------------------------|------|----------------|-------------|--------------|--------------|
| 46,X,dir dup(X)(q11.2-q22.3) | + | + | + | ++ | + |

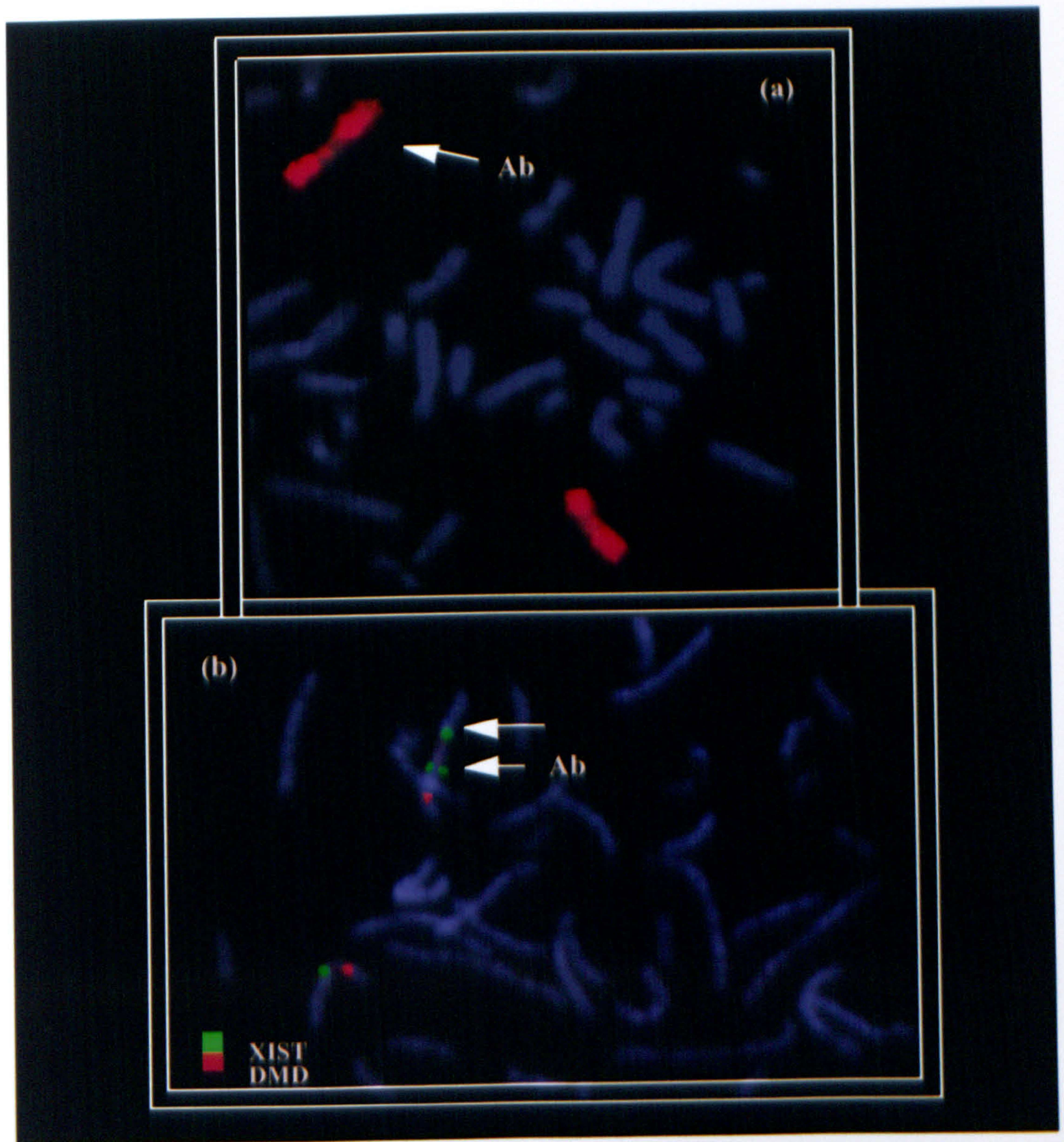


Figure 3.18: FISH investigation on metaphase spreads from a 46,XXq+ patient. (a) Hybridisation of whole X chromosome library revealed a complete coverage on both chromosomes. (b) A dual FISH study using DMD (Xp21) and XIST (Xq13) probes showed two copies of XIST locus on the abnormal X chromosome (arrows).
Ab= abnormal

Case 11

A 16 years old female was referred due to clinical features of Turner syndrome. Routine cytogenetic analysis using G-banding showed mosaicism for X chromosome. Four out of 25 cells showed 45,X karyotype and the remaining showed a count of 46, with one normal X chromosome and one X chromosome with additional material on the distal long arm at Xq28 with banding pattern suggesting an inverted duplication of the region (X)q28→22.1. Her karyotype was interpreted as mos 45,X/46,X,dup(X)(q28 →q21.1).

Slides from this case were hybridised with whole chromosome library for the X chromosome and showed a complete coverage of the normal and abnormal X chromosomes (Figure 3.19b). Slides were then hybridised to a panel of X chromosome specific probes, DXZ1 (centromere), DMD (Xp21), XIST (Xq13) and DXZ4 (Xq24). FISH analysis of case 11 revealed two copies of the DXZ4 and one copy of XIST loci on the long arm of the abnormal X chromosome and one copy of DMD locus on the short arm (Figure 3.19c and d). These results confirmed duplication of Xq22.1→Xq28.

Unfortunately, due to lack of material and probes it was not possible to confirm precisely the type or the nature of duplication (either direct or inverted duplication of Xq). This could have been confirmed by using probes in the region Xq25-28 or telomeric. The FISH analysis findings of case 11 are summarised in the inserted table.

| Case # 11 | WCPX X-paint | DXZ1 X-cent | DMD Xp21 | XIST Xq13 | DXZ4 Xq24 |
|-------------------------------------|-----------------|----------------|-------------|--------------|--------------|
| 45,X[10]/46,X,dup(X)(q22.1-q28)[90] | + | + | + | + | ++ |

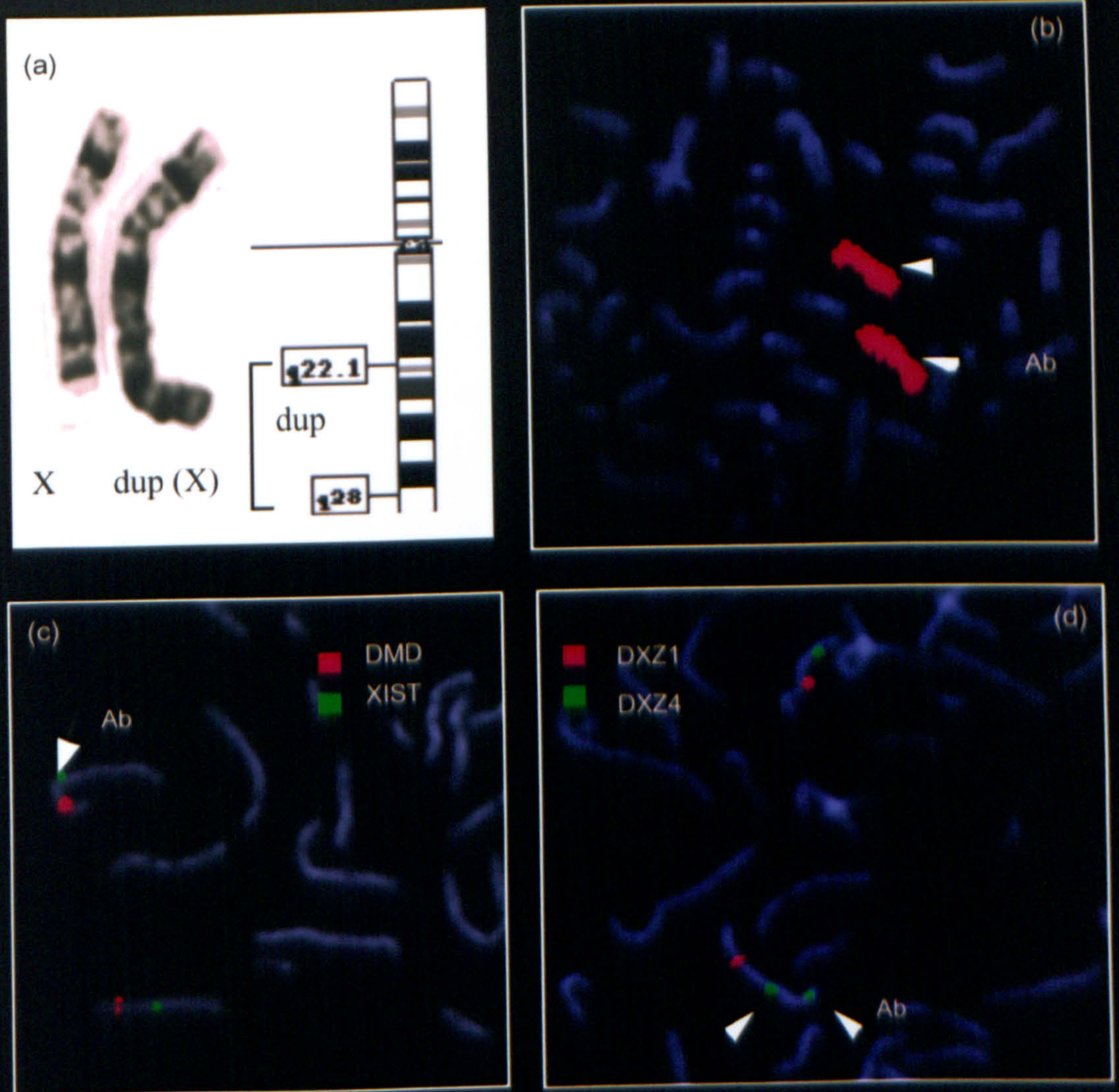


Figure 3.19: FISH study using different X specific probes on metaphase spreads from a patient with a 45,X/46,XXq+ karyotype. a) G-banded X chromosomes and diagram of X chromosome showing the dup(X) region. b) Whole X chromosome library showing complete coverage of both chromosomes. c) Dual FISH using DMD and XIST probes showing one copy of each probe on both chromosomes. d) Dual FISH results using DXZ1 and DXZ4 revealed two copies of DXZ4 (Xq24) signals on the abnormal X (arrows). Ab = abnormal X chromosome, dup = duplication.

(3) FISH study of 46,X,? i(Xp)

Two cases with Turner phenotype and with cytogenetic findings of 46,X,?i(Xp) and 46,XX/46,X,?i(Xp) were investigated by the FISH technique to identify the status of the presumed isochromosome.

Case 12

This female was referred with Turner phenotype. Routine cytogenetic analysis using G-banding showed 46,X,i(Xp) karyotype.

FISH analysis of 100 metaphase spreads using X-centromeric (DXZ1) and whole X chromosome paint on old slides (~ 4 months old) revealed no mosaicism in this patient. FISH analysis using probes specific for the short and the long arms of the X chromosome were applied. As can be seen from Figure 3.20, this case was shown to contain one copy of the DMD locus (Xp21.2) on each arm of the abnormal X chromosome and no signal for the FRAXA/E probe was detected. The XIST probe was also applied and clear signals were observed on the long arm of the normal chromosome, however, the XIST signal could not be delineated precisely on the abnormal X in the 15 cells analysed due to overlapping or superimposing of DXZ1 probe (Figure 3.20d). The superimposing of DXZ1 over the XIST signal could be due to the condensed chromosome seen in these slides. However, this observation was not detected in any studied cases by the XIST probes. Therefore, the interpretation of i(Xp) may be incorrect in this instance. The possibility of Xp;Xp translocation has been raised. This case has been recently reinvestigated by another PhD student in the department DGI (Glasgow), and it has been proven that this case is not an i(Xp) (Dr. Boyd personal communication, 1999).

| Case 12 | DXZ1 X-cent | DMD Xp21 | XIST Xq13 | FRAXA/E Xq27-28 |
|------------|----------------|-------------|--------------|--------------------|
| Normal X | + | + | + | + |
| Abnormal X | + | ++ | ?* not clear | - |

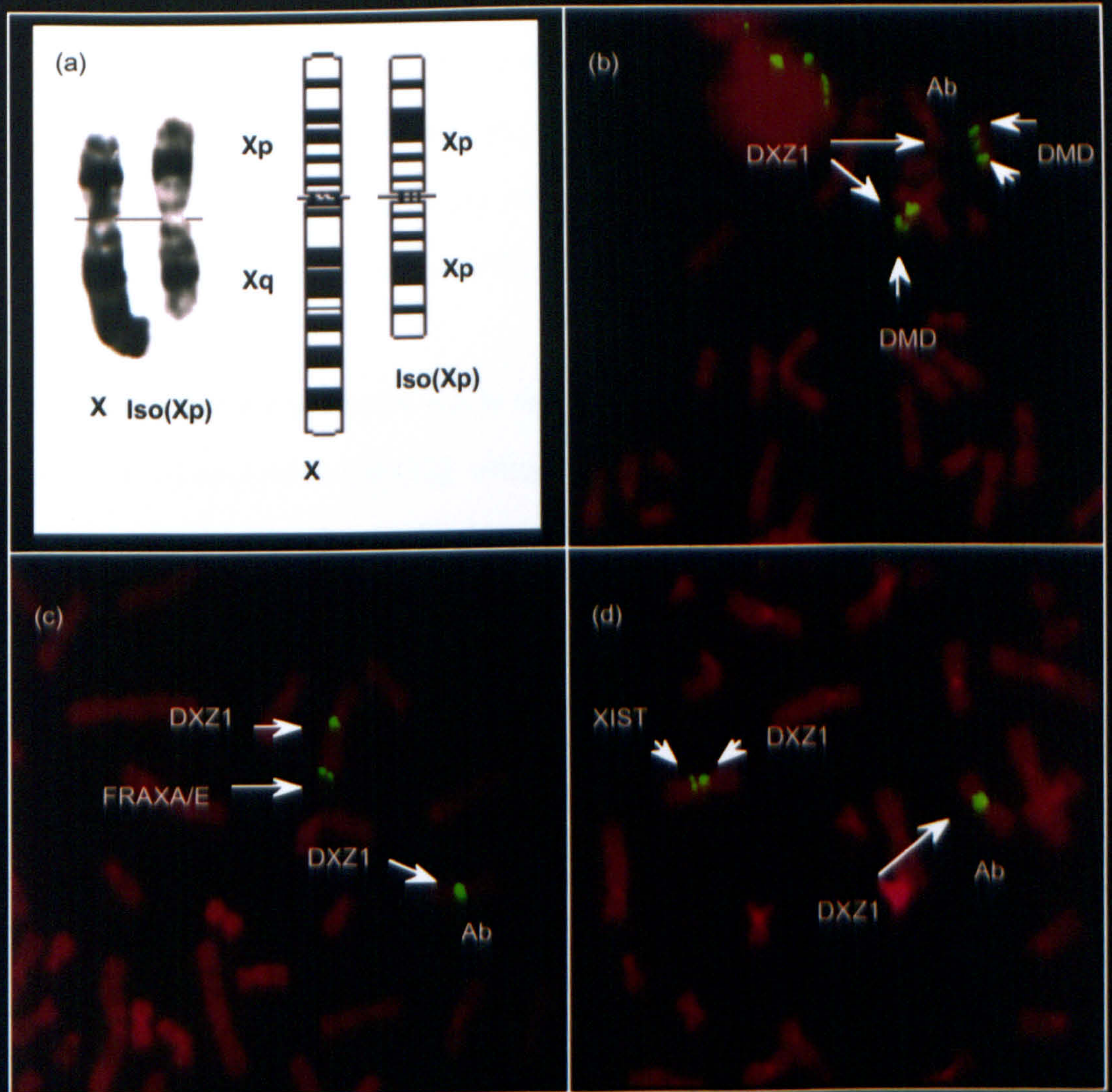


Figure 3.20: (a) G-banded X chromosomes of a patient with 46,X,i(Xp) karyotype and an ISCN diagram of normal X chromosome and Iso(Xp). (b) FISH study using DMD (Xp21) probe. One copy of the DMD locus detected on each arm of the abnormal X chromosome (arrows). No signals for the FRAXA/E and XIST probes were detected on the abnormal X chromosome although clear signals of both probes were obtained on the normal X chromosome (c-d). All probes were co-hybridised with DXZ1 (X centromeric probe). Ab = abnormal.

Case 13

This 8 year old female was referred with Turner phenotype. Routine cytogenetic study using G-banding confirmed the presence of two cell lines. The first cell line showed an apparently normal 46,XX karyotype. The second cell line showed one normal X chromosome and one structurally abnormal X chromosome. Her karyotype was interpreted as mos 46,XX/46,X,?i(Xp) in the ratio of 52% and 48% respectively.

FISH analysis was carried out to delineate the structural rearrangement of the derivative X chromosome [der(X)] using probes DXZ1, DMD, KAL, XIST, and DXZ4. FISH results in this mosaic case revealed one copy of both DMD and XIST probes on the normal X and the abnormal X chromosomes. Hybridisation with KAL (Xp22) probe demonstrated two copies of KAL signals one on each arm of abnormal X and one copy of KAL on the short arm of the normal X chromosome. In addition, hybridisation with DXZ4 probe (Xq24) to the same case showed clear signals on the long arm of the normal X chromosome, while no signals could be detected on the abnormal X chromosome (Figure 3.21). The in situ hybridisation studies demonstrated this abnormal X chromosome to be a derivative chromosome arising from a translocation between two X chromosomes with the breakpoints at p22.1 and q22, resulting in monosomy for the region (X) q22→Xq ter and trisomy for the region (X) p22→Xp ter in this cell line. These studies thus reinterpreted the karyotype to 46,XX/ 46,X,der (X) t (X;X) (p22.1;q22).

| Case 13 | KAL (Xp22) | DMD (Xp21) | DXZ1 (X- cen) | XIST (q13) | DXZ4 (q24) |
|------------------------------|---------------|---------------|------------------|---------------|---------------|
| 46,XX[58]/46,derX,t(X;X)[42] | | | | | |
| Normal X | + | + | + | + | + |
| Abnormal X | ++ | + | + | + | - |

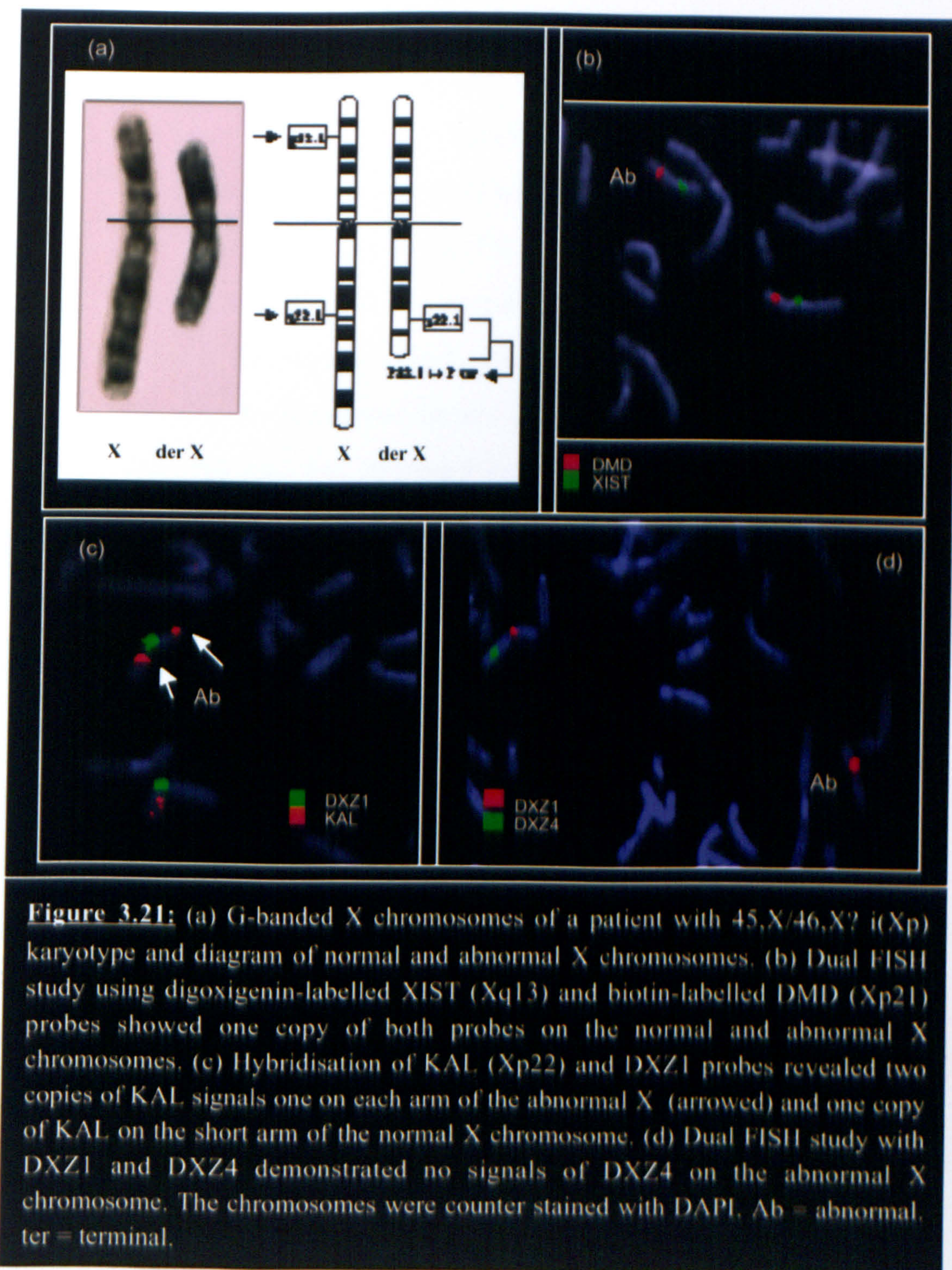


Figure 3.21: (a) G-banded X chromosomes of a patient with 45,X/46,X?i(Xp) karyotype and diagram of normal and abnormal X chromosomes. (b) Dual FISH study using digoxigenin-labelled XIST (Xq13) and biotin-labelled DMD (Xp21) probes showed one copy of both probes on the normal and abnormal X chromosomes. (c) Hybridisation of KAL (Xp22) and DXZ1 probes revealed two copies of KAL signals one on each arm of the abnormal X (arrowed) and one copy of KAL on the short arm of the normal X chromosome. (d) Dual FISH study with DXZ1 and DXZ4 demonstrated no signals of DXZ4 on the abnormal X chromosome. The chromosomes were counter stained with DAPI. Ab = abnormal, ter = terminal.

(4) FISH result of i(Xq)

Case 14

Fixed material from phytohaemagglutinin (PHA) stimulated blood culture was obtained from a Turner syndrome patient. Chromosome analysis by G banding showed the presence of 3 cell lines. The first had 45,X karyotype in 3 out of 30 cells examined, while the second had a 46 count with one normal X chromosome and one isochromosome for the long arm of the X in 25 out of 30 cells examined. The third cell line has a count of 47 with one normal X chromosome and two copies of the isochromosome for the long arm of the X in 2 out of 30 cells examined. A molecular study of her DNA by Dr. Chu showed the presence of Y chromosome (Yq) sequences. For that reason, this case was forwarded for FISH study to determine whether low grade mosaicism of Y chromosome was present.

The status of the abnormal X chromosome and the percentage of mosaicism was confirmed by FISH experiments with X-centromere and X-paint probes. The whole X-paint showed a complete coverage of the normal X chromosome as well as the abnormal X (iXq). No translocation was detected on any other chromosome. X-centromere probe DXZ1 revealed the presence of two copies of signals on the abnormal chromosome (dicentric) and one copy of DXZ1 on the normal X chromosome (Figure 3.22). Moreover, X chromosome paint for the long arm (CAP) revealed complete coverage of both arms of the abnormal X chromosome confirming the cytogenetic diagnosis of i(Xq) of the abnormal chromosome. FISH analysis using Y specific probes (PDP97, PHY2.1, Y-paint) on metaphase spreads of this patient showed no signals with any of the panel of Y chromosome specific probes. The molecular cytogenetic (FISH) results failed to detect the presence of Y material in this patient and confirmed the cytogenetic finding of the patient with a karyotype of 45, X / 46, X, i dic (Xq) / 47, X, i dic(Xq) i dic (Xq) in the ratio of 12%, 84%, and 4% respectively.

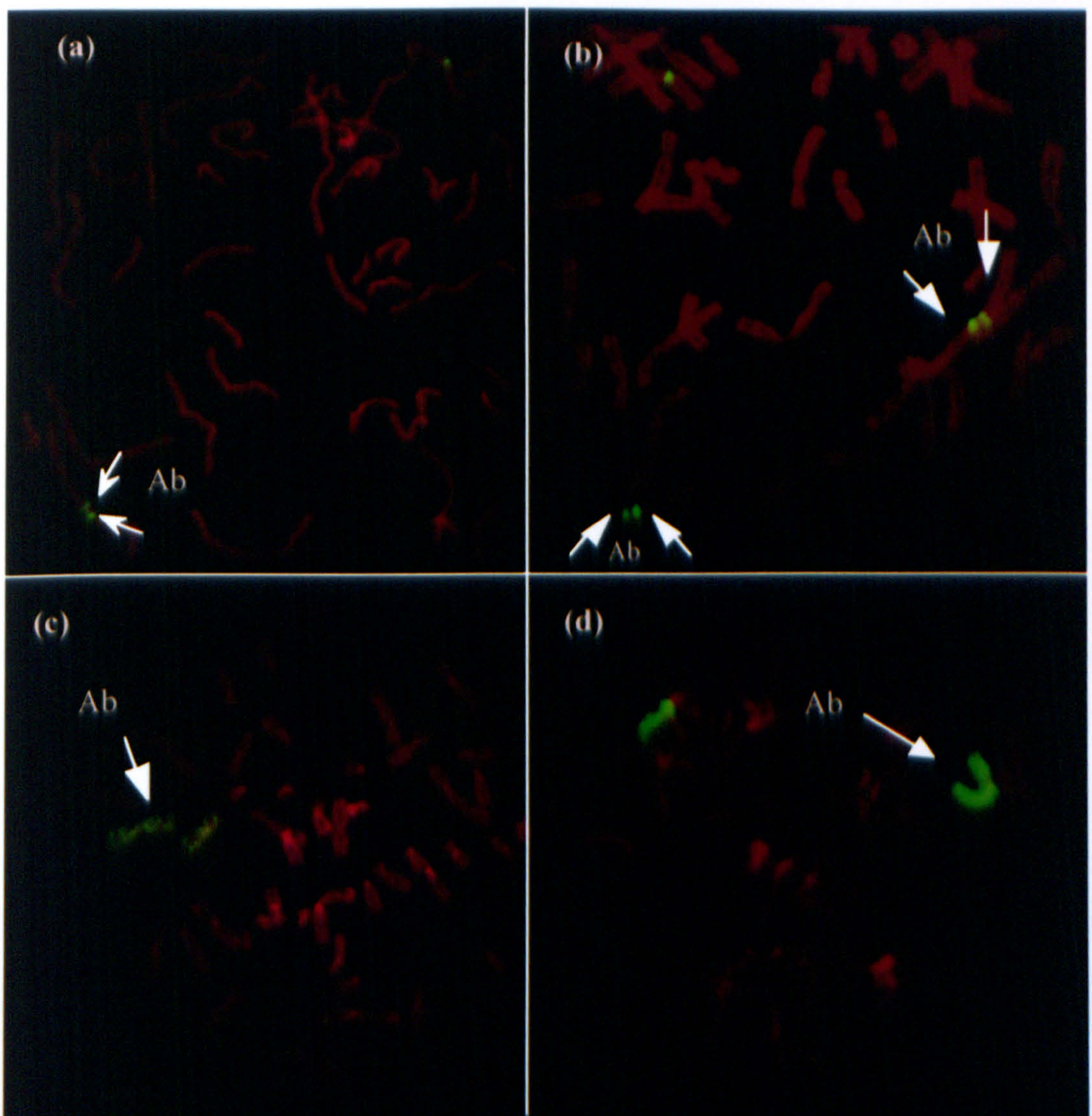


Figure 3.22: FISH study using DXZ1, whole X chromosome library (paint) and chromosome arm paint (CAP) of the long arm of the X chromosome on metaphase spreads of a patient with a karyotype of 45,X/46,X,i(Xq)/47,X,i(Xq),+i(Xq). (a, b) Hybridisation with DXZ1 showed two copies of the probe signals at the centromeric region (dicentric) of the abnormal X chromosomes. (c) FISH analysis with X paint showed a complete coverage of the paint over the normal and the abnormal X chromosomes. (d) FISH application of the long arm of the X chromosome paint (CAP) revealed a complete coverage of the probe over the abnormal chromosome confirming the cytogenetic finding of i(Xq) cell line in the patient. Ab = abnormal.

(5) FISH results from Turner syndrome with Xp deletion

Case 15

This female was referred with clinical features of Turner syndrome. The cytogenetic studies using Giemsa banding technique of this Turner patient revealed that she had a karyotype of 46,XXp- (pter→p11). FISH analysis using DXZ1 probe on metaphase spreads of her peripheral blood lymphocytes showed hybridisation signals at the centromeric region of the normal chromosome and on a large acrocentric like chromosome. Application of the X-paint demonstrated a complete coverage of the X-paint over the normal X and the acrocentric like chromosome (Figure 3.23). FISH study using KAL probe specific for the short arm of X chromosome on metaphase spread from patient's blood samples demonstrated the presence of the KAL probe only on the short arm of the normal X. Moreover, X chromosome paint for the long arm (CAP) revealed complete coverage of the paint over the abnormal X indicating that the abnormal chromosome contained only Xq material. Hybridisation analysis studies thus demonstrated that the short arm of the abnormal X chromosome was entirely deleted confirming the karyotype to be 46,XXp-. Summary of FISH analysis is shown in the table below.

| Case 15 | DXZ1 | WCPX | KAL (Xp22) | CAP Xq |
|----------|------|------|---------------|--------|
| Normal X | + | + | + | + |
| Xp- | + | + | - | + |

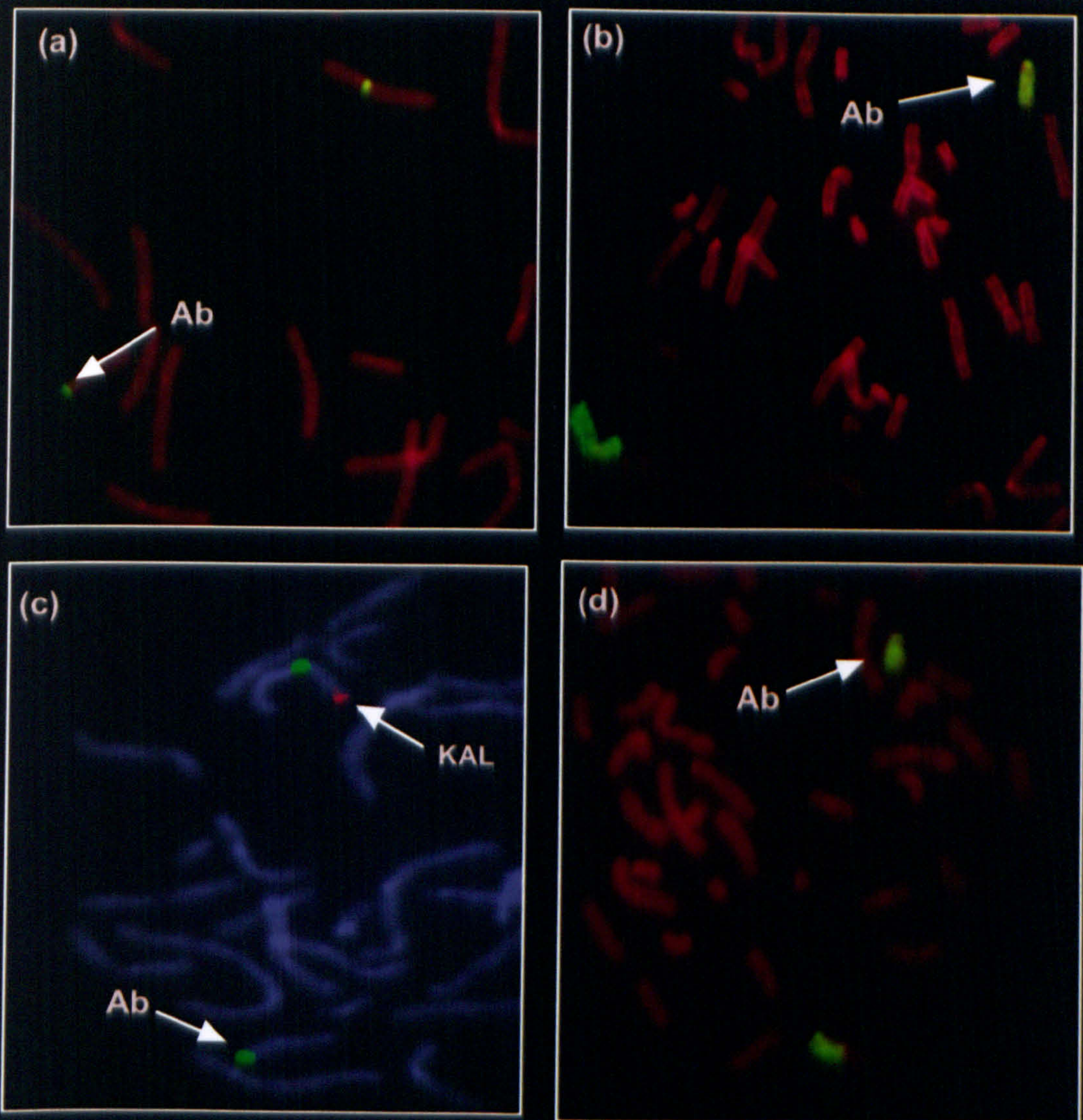


Figure 3.23: FISH investigation of 46,XXp- case. (a) Application of DXZ1 showed hybridisation at the centromeric region of normal X and the abnormal X (acrocentric like) chromosomes. (b) Whole X library probe FISH study showed a complete coverage of the paint over both X chromosomes. (c) Dual FISH study using KAL and DXZ1 showed signals only on the short arm of the normal X chromosome (arrow). (d) Long arm DNA painting of the X chromosome showed coverage of the long arm of the normal X and complete coverage of the abnormal X. These results confirmed the cytogenetics findings of the patient with deletion of the short arm of the X chromosome. Ab = abnormal.

(6) FISH investigation of a short stature Female

Case 16

A 16 years old female who presented at 9 years of age with short stature, solitary right kidney, clitoral hypertrophy, urogenital sinus with bladder diverticulum and enuresis. She was later found to have an absent left ovary also. Chromosomal analysis of 20 metaphases showed an apparently normal 46,XX karyotype. This case was referred for FISH investigation to exclude the possibility of 46, XX/ 46, XY or 45,X/46,XX mosaicism. FISH studies on 100 metaphase cells and 100 interphase nuclei of the patient with Y chromosome specific probes, PHY2.1, PDP97, and WCPY (Y-paint) revealed that the patient was negative for all three tested probes. FISH study using DXZ1 and WCPX (X-paint) probes specific for chromosome X on 100 mitoses demonstrated two cytologically normal X chromosomes. These findings indicated that the patient has a 46,XX karyotype with no detectable aneuploidy either for X or Y chromosomes.

| Case 16 | WCPX (X-paint) | DXZ1 (X-cen) | WCPY (Y-paint) | PDP97 (Y-cen) | PHY2.1 (Yq12) |
|--------------------------------|---------------------------|-------------------------|---------------------------|--------------------------|--------------------------|
| 46,XX short stature | ++ | ++ | - | - | - |

(7) FISH analysis of 45,X/ 46, XY Turner syndrome

Cases 17 and 18

Both female cases presented at a few months of age with failure to thrive and went on to have short stature. Both had gonadectomy at 6 years and 2 years of age respectively. Routine cytogenetic analysis of 50 G-banded metaphases confirmed mosaicism 45,X/46,XY in both cases in the ratio of 40%, 60% in case 17 and 52%, 48% in case 18.

Fluorescence *in situ* hybridisation was performed on those two cases using centromeric probes and whole chromosome paints of chromosomes X and Y. The percentage of mosaicism using interphase nuclei and metaphase FISH study revealed 48% of the 45,X cell line in one case and 42% in the other case. No structural aberrations of chromosomes X or Y were detected. Thus the status of the cytogenetic finding is confirmed by FISH.

| Case No | Karyotype | DXZ1 | WCPX | WCPY | PDP97 (DYZ3) |
|---------|------------|------|------|------|-----------------|
| 17 | 45,X [42] | + | + | - | - |
| | 46,XY [58] | - | - | + | + |
| 18 | 45,X [48] | + | + | - | - |
| | 46,XY [52] | - | - | + | + |

(8) A case with marker chromosome

Case 19

A female aged 21 years old presented with Turner phenotype. Chromosomal analysis by G-banding revealed the presence of two cell lines. One cell line showed a 45,X karyotype and the second showed a 46,X, + a small marker chromosome of unknown origin in 6% of her cells. DNA analysis by PCR had been carried out and confirmed the presence of Y-specific sequences. Dual FISH study using DXZ1 and PDP97 centromeric X and Y probes respectively on metaphase chromosomes revealed two copies of PDP97 hybridisation signals on the marker chromosome (dicentric, Figure 3.24). Dual FISH using biotin labelled WCPY and digoxigenin labelled WCPX paints detected the presence of both X and Y sequences on the marker chromosome. The Y sequence was sandwiched between X material suggesting X-Y translocations leading to isodicentric t(X;Y) (Figure 3.24). These signals are most likely to be real hybridisation and not due to cross hybridisation as they could not be detected in normal male control. Therefore, the karyotype of this case can be interpreted as 45,X/46,X,i dic t(X;Y) in the ratio of 96% and 4% respectively. Unfortunately, due to small amount of sample, further investigation-using panels of both X and Y specific probes could not be carried out to delineate the rearrangement more precisely.

| Case 19 | WCPX | DXZ1 (X-cen) | WCPY | PDP97 (DYZ3) |
|-----------------------|-------------|-------------------------|-------------|-------------------------|
| 45,X [96] | + | + | - | - |
| 46,X,+ mar [4] | ++ | + | ++ | ++ |



Figure 3.24: In situ hybridisation study of a Turner syndrome patient with a 45,X/46,X,+mar karyotype. (a) Dual FISH study using DXZ1 and PDP97 probes specific for centromeric regions of X and Y chromosomes respectively. Two copies of PDP97 signals were obtained on the marker chromosome (arrows). (b) FISH analysis using whole chromosome libraries for the X and Y showed hybridisation signals of both paints on the marker chromosome. The X paint was shown to be present at both arms of the marker chromosome (arrows).
mar = marker.

(9) FISH study of a case with 46,XXp+

Case 20

A female presenting with dysmorphic features, developmental delay, epilepsy and anal abnormalities was referred to the cytogenetic laboratory to investigate her chromosomal constitution. G-banding technique revealed an abnormal female karyotype 46,XXp+. This case was then investigated by FISH technique bearing in mind that the extra material could be from chromosome 22 based on some of her clinical features such as heart defect, anal abnormalities, mental retardation and dysmorphic features.

FISH analysis using whole chromosome libraries for X and 22 chromosome (Cambio) showed a normal hybridisation pattern of both probes (Figure 3.25a and b). No translocation or deletion could be detected using these two probes. This result implied that the extra material could originate from an X chromosome.

The slides were then hybridised to a panel of X-specific probes. Two different probes specific for the short arm of the X chromosome were used (KAL and Xp-telomere) and one signal for each probe was observed on the short arms of both normal and abnormal X chromosomes (Figure 3.25c and d). However, it was noticed that the Xp-telomeric probe on the abnormal X chromosome consistently showed reduced signal intensity in comparison to normal X chromosome. Also there is material distal to it.

Three specific probes for the long arm of the X chromosome were also applied (FRAXA/E, DXZ4 and Xq-telomere). Normal hybridisation signals were detected on the normal and abnormal chromosomes (Figure 3.25e-g).

Recently, chromosome arm DNA painting (CAP) probes specific for the X chromosome were obtained and applied to metaphase spreads from this patient. Application of Xp CAP showed complete coverage of the paint on the short arm of the normal X chromosome and a partial coverage on the short arm of the abnormal X chromosome. FISH study using Xq CAP revealed complete coverage on the long arms of both normal and abnormal X and no signal was observed on the short arm of the X chromosomes (Figure 3.25h-i). These results indicated that the extra material could be of autosomal or Y chromosomal origin and that there was a misleading result obtained upon using the whole X chromosome library (Figure 3.25a).

The fixed materials were then hybridised to individual chromosome library probes for all autosomes and sex chromosomes using the Cytocell Multiprobe Kit (Cytocell), and the extra material on the short arm of the abnormal X chromosome was shown to have originated from chromosome 16 (Figure 3.26). This result was reconfirmed upon the application of Cambio whole chromosome 16 library. FISH study using chromosome arm paint (CAP) specific for the short arm and long arm of chromosome 16 was applied and revealed that the extra material on the X chromosome originated from the long arm of chromosome 16 (16q) (Figure 3.26b). Therefore comprehensive FISH analysis confirmed X:16 translocation in this patient, leading to trisomy 16q with final karyotype of [46,X,-X,+ t(X;16)(p22.3;q24]. Detailed FISH analysis is summarised in the table below.

Case 20: 46,XXp⁺ [t(X;16)]

| Probes | Observation | |
|------------------------|--|---|
| | Normal X | Abnormal X [der(X)] |
| WCPX (CAMBIO) | + | + |
| WCP22 (CAMBIO) | + | + |
| DXZ1 (X-cen) | + | + |
| KAL (Xp22) | + | + |
| Xp tel | + | +/- (reduced intensity) |
| DXZ4 (Xq22) | + | + |
| FRAXA/E (Xq27-28) | + | + |
| Xq tel | + | + |
| CAP Xp | + | +/- (incomplete coverage) |
| CAP Xq | + | + |
| Cytocell MPK(1-22,X,Y) | There were 3 signals for 16, one on the abnormal X chromosome | |
| WCP16 (CAMBIO) | Confirmed 3 copies of chromosome 16q, one on the abnormal X chromosome | |
| CAP 16p | - | - |
| CAP 16q | - | There were 3 signals for 16q, one on the short arm of the abnormal X chromosome |

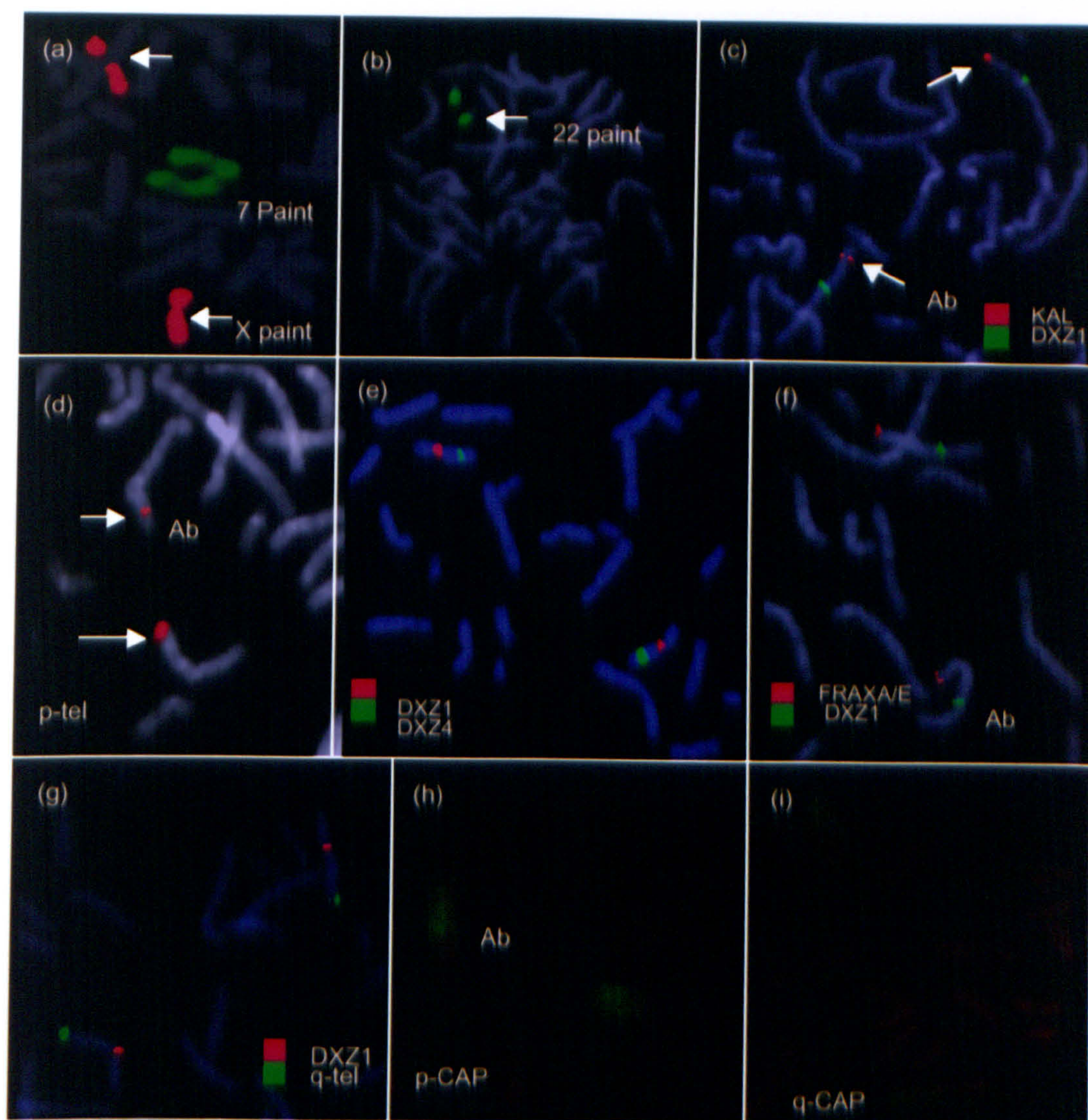


Figure 3.25: FISH study of a patient with 46,XXp+ karyotype. Hybridisation with X paint showed a complete coverage of the paint over both X chromosomes (a). Whole chromosome 22 paint showed a complete coverage of the paint over both chromosomes 22, and no translocation or deletion was detected (b). Metaphase chromosomes then were hybridised to a panel of probes specific for the X chromosome. Application of KAL (c) and p-telomere (d) probes showed one copy of each probe on both normal and abnormal X chromosomes, however the p-telomere was less in intensity and showed more chromosome material distal to the signal of the abnormal X chromosome compared to the normal X chromosome. Application of DXZ4 (e), FRAXA/E (f) and Xq-telomere (g) showed one copy of each probe on both X chromosomes. FISH study using p-CAP showed partial coverage over the short arm of the abnormal X chromosome (h). Application of q-CAP showed a complete coverage over the long arms of both X chromosomes (i). Ab = abnormal.

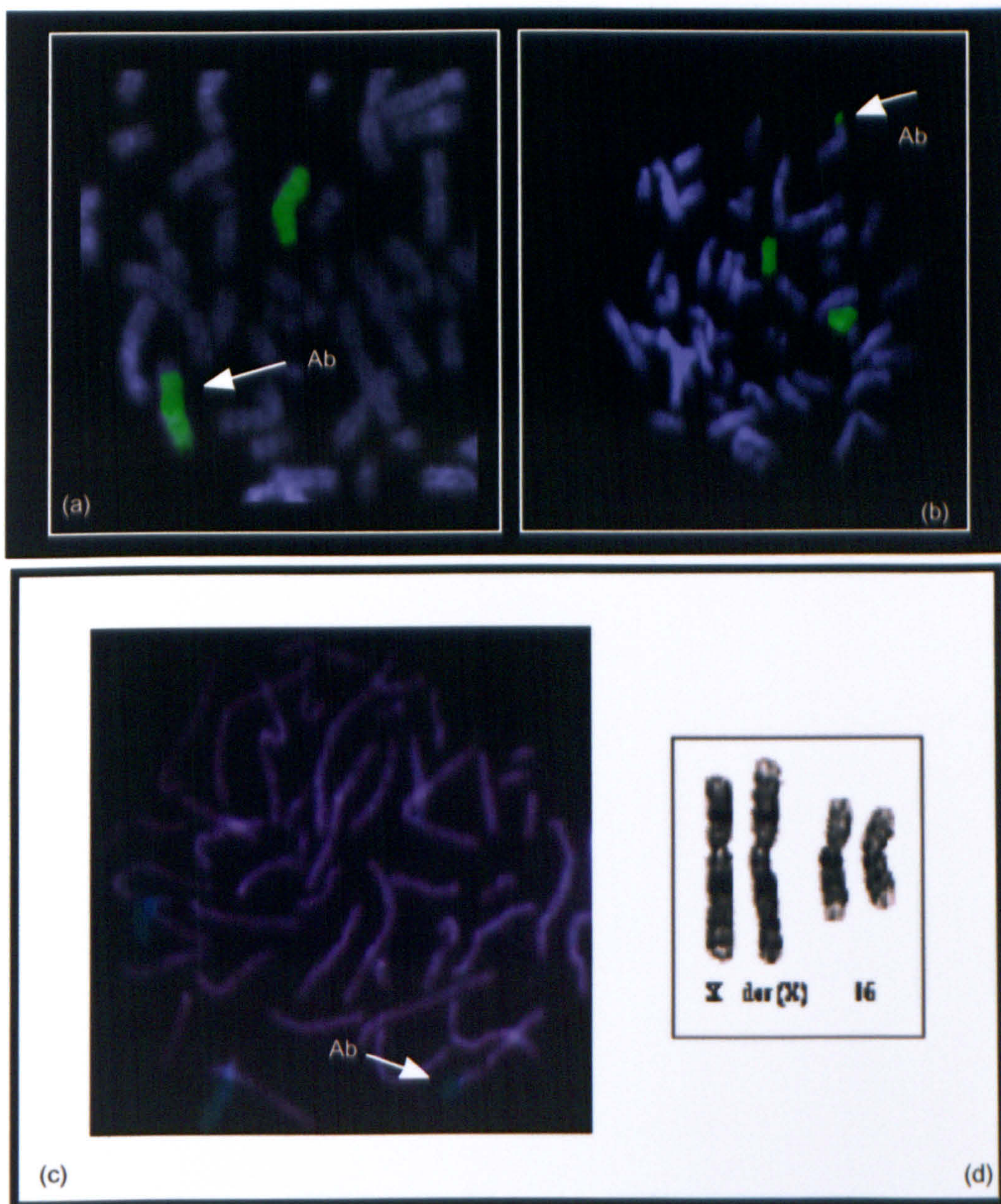


Figure 3.26: Application of the Cytocell Multi probe revealed a complete coverage of the Xpaint over the normal and a partial coverage over the abnormal X chromosome (a). Whole 16 library showed a complete coverage of the paint over both chromosome 16 and extra hybridisation signal was detected on the abnormal X chromosome (b). FISH study of 16q-CAP showed complete coverage of the q arm of chromosome 16 and the p arm of the abnormal X chromosome (c). Partial G-banded karyotype showing the derivative X {t(X;16)}, normal chromosomes X and 16 (d). Ab = abnormal.

(B) FISH studies of cases with Y chromosome abnormalities

Case 21

A phenotypically normal female with primary amenorrhea was cytogenetically investigated and showed a 46,X,t(Xp;Yq) karyotype.

FISH analysis using Y-paint probe to metaphase spreads of this case showed the presence of hybridisation of Y sequences on the abnormal X chromosome (Figure 3.27a). FISH analysis using DXZ1 and PDP97 probes to metaphase spreads showed that the abnormal X was positive for the X-centromeric probe (DXZ1) and was negative for PDP97 probe (Figure3.27b). Hybridisation study using GMGY10 and SRY probes (both hybridise to the TDF region) revealed no signals of these probes in either metaphase or interphase nuclei. Y-chromosome long arm-specific probes (MK5 and PHY2.1) hybridisation studies showed clear and strong signals of these probes on the short arm of the abnormal chromosome X (Figure 3.27b, c, d). Dual FISH analysis using XIST (Xq13) and PHY2.1 (Yqh) showed clear signals of both probes on the abnormal X chromosome and revealed that the abnormal X chromosome carries the Y material on the short arm (Figure 3.27d). These findings of FISH analyses reconfirmed the cytogenetic finding of translocation of Yq to the short arm of the X chromosome and showed the absence of the TDF region in the abnormal X.

| Case 21 | WCP-Y Y-paint | DXZ1 (X-cen) | PDP97 (Y-cen) | MK5 (Yq11) | PHY2.1 (Yq12) | XIST (Xq13) | GMGY10 (Yp11) | SRY (Yp11) |
|--------------------|--------------------------|-------------------------|--------------------------|-----------------------|--------------------------|------------------------|--------------------------|-----------------------|
| 46,X,t(X;Y) | + | + | - | + | + | + | - | - |

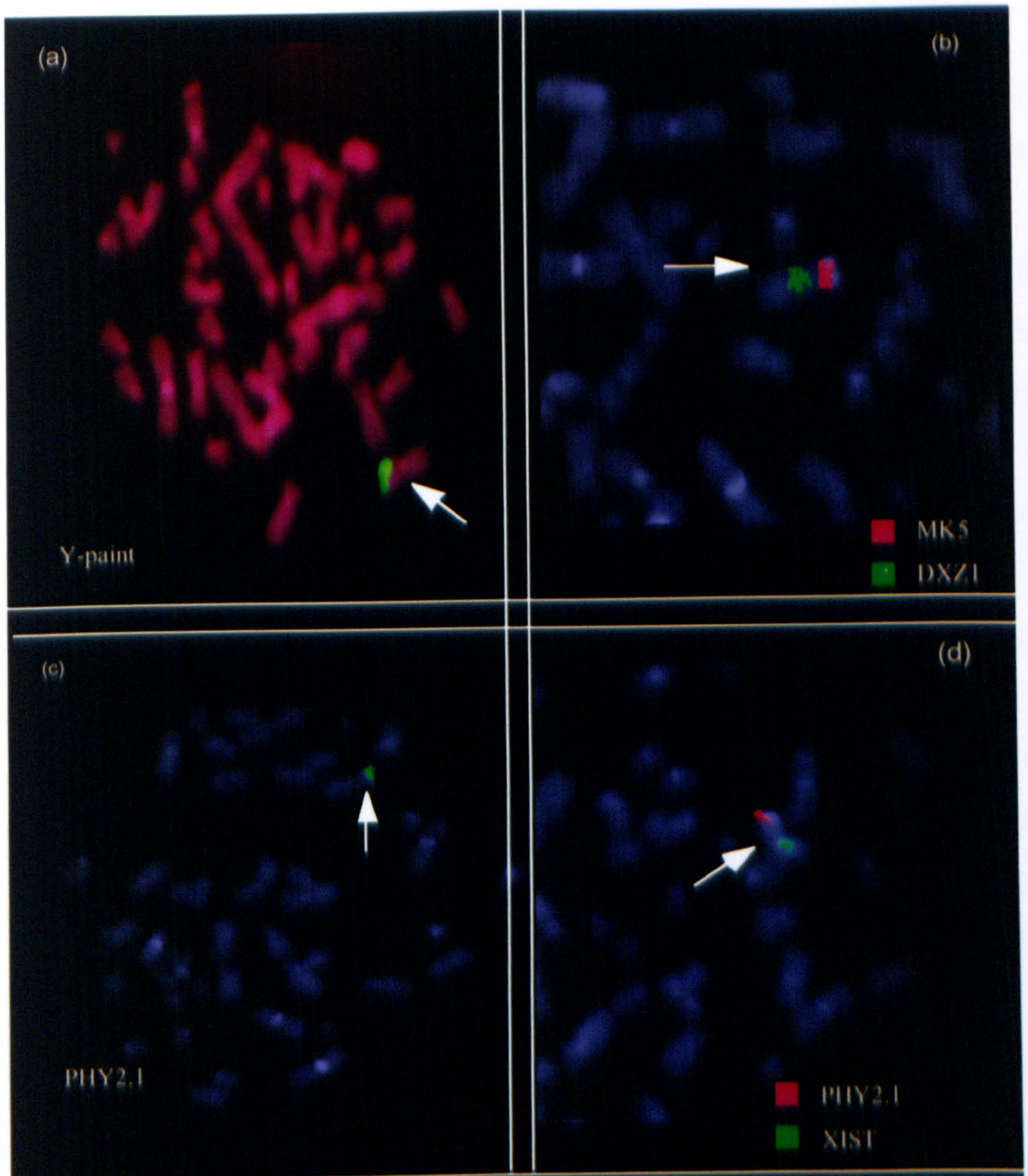


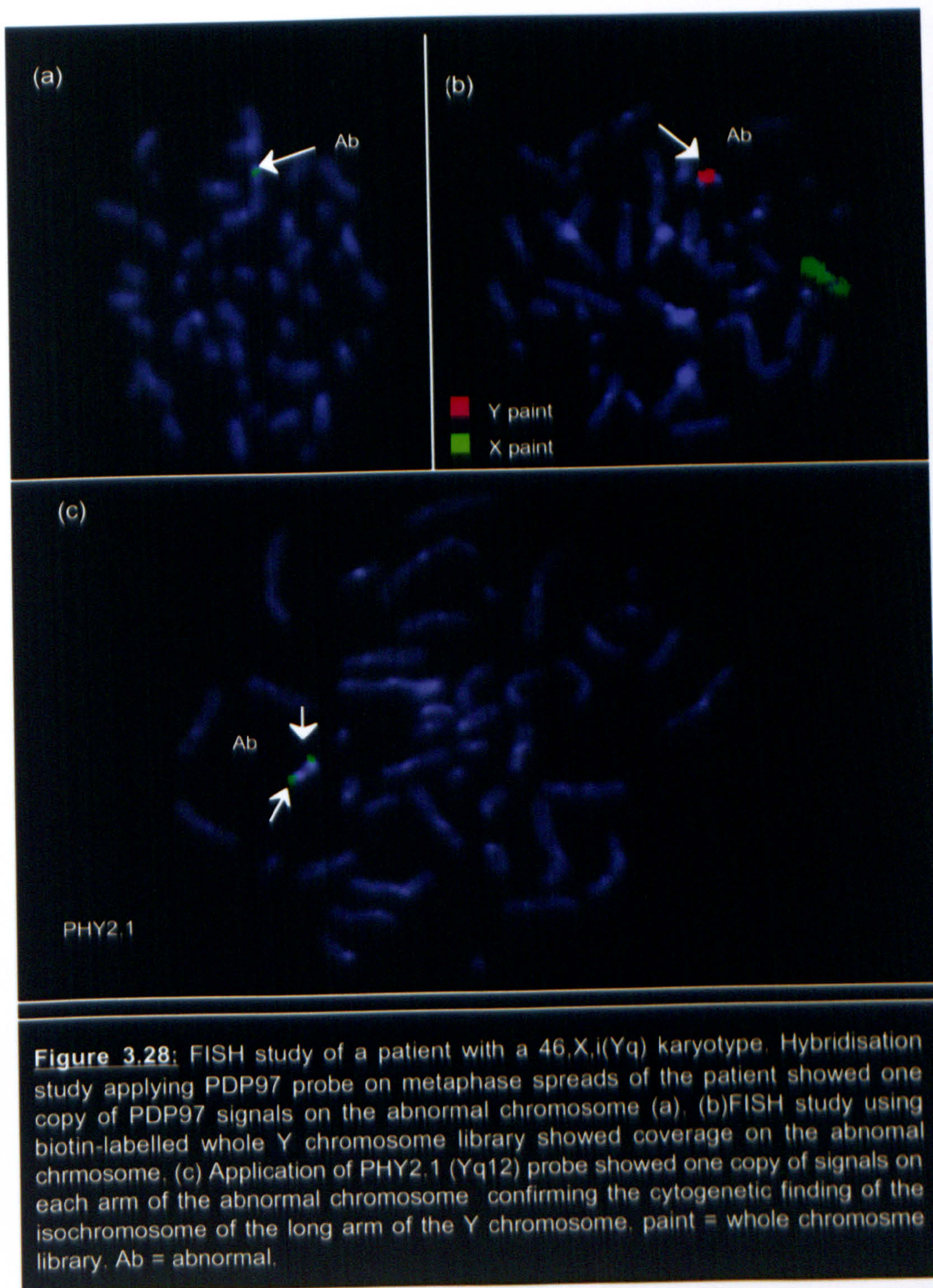
Figure 3.27: FISH analysis on metaphase spreads of a patient with a 46,X,t(Xp;Yq) karyotype. (a) Hybridisation with whole chromosome library of the Y showed signals on the abnormal chromosome. (b) Dual FISH study using DXZ1 (X-centromere) and MK5 (Yq11.2) showed one copy of both probes on the abnormal chromosome. (c) Hybridisation of PHY2.1 (Yq12) revealed signals on the abnormal chromosome. (d) Application of XIST (Xq13) probe on metaphase spreads from the same patient showed clear signals on the abnormal chromosome.

Case 22

A female referred with history of primary amenorrhea was investigated. Cytogenetic analysis using G-banding technique confirmed her karyotype as mos 45,X/46,X,iY(q) in the ratio of 74% and 26% respectively.

FISH study to metaphase spreads using PDP97 probe showed 70% of the examined cells to be negative for this probe confirming the presence of 45,X cell line, while 30% of the cells showed hybridisation signal of the Y chromosome (Figure 3.28a). Dual FISH study using both X and Y chromosome paint (WCP X and Y) probes and unicolour FISH study using Y chromosome long arm-specific probe (PHY2.1) revealed X-paint coverage over the normal X chromosome and Y paint on the abnormal Y chromosome (Figure 3.28b) and two copies of the PHY2.1 signals at both arms of the abnormal Y (Figure 3.28c). Moreover, hybridisation with Y-chromosome short arm-specific probes (GMGY10, SRY) showed no signals on the abnormal Y indicating absence of Yp material in the abnormal Y chromosome. These results confirmed the cytogenetic interpretation of the karyotype, as 45,X/46,X,i(Yq).

| Karyotype | PDP97 Y-cent | WCPX | WCPY | PHY2.1 Yq12 | SRY Yp11 | GMGY10 Yp11 |
|-----------------|-----------------|------|------|----------------|-------------|----------------|
| 45,X (67%) | - | + | - | - | - | - |
| 46,X,i(Yq)(33%) | + | - | + | + + | - | - |



Case 23

A male patient with ambiguous genitalia with the karyotype mos 46,XY/46,X,+mar (?derY) was reassessed to delineate the status of the abnormal Y chromosome material. FISH analysis using centromeric probe (PDP97) revealed that the abnormal Y chromosome was positive for Y centromere. However dual colour FISH using biotin labelled WCPY paint and digoxigenin labelled WCPX paint revealed a complete Y-paint coverage over the normal and abnormal Y chromosome (Figure 3.29). Dual colour FISH analysis using GMGY10 (Yp11) and PHY2.1 (Yq12) probes revealed one copy of each probe adjacent to each other on the abnormal Y chromosome (Figure 3.29) suggesting the possibility of a ring Y chromosome [r(Y)] . Dual colour FISH analysis using PDP97 and MK5 (Yq11) probes showed one copy of apparently large Y centromeric probe (PDP97) sandwiched between two copies of the MK5 probe (Figure 3.29) suggesting the possibility of dicentric Yq or isodicentric Yq. Therefore, FISH analysis confirmed the karyotype of the patient contain multiple cell lines mosaicism involving structural abnormalities of the Y chromosome. Cytogenetic reassessment of the case using G, C, and Q banding reconfirmed the FISH results. The revised karyotype was interpreted as mos 46,XY[91]/46,X,i(Yq)[6]/46,X,r(Y)[3] (Figure 3.30).

| Karyotype | WCPX | WCPY | GMGY10 (Yp11) | PHY2.1 (DYZ2) | MK5 (Yq11) | PDP97 (DYZ3) |
|---------------|------|------|------------------|------------------|---------------|-----------------|
| 46,X,i(Yq)[6] | - | + | Not done | Not done | ++ | + |
| 46,X,r(Y)[3] | - | + | + | + | N.D | + |

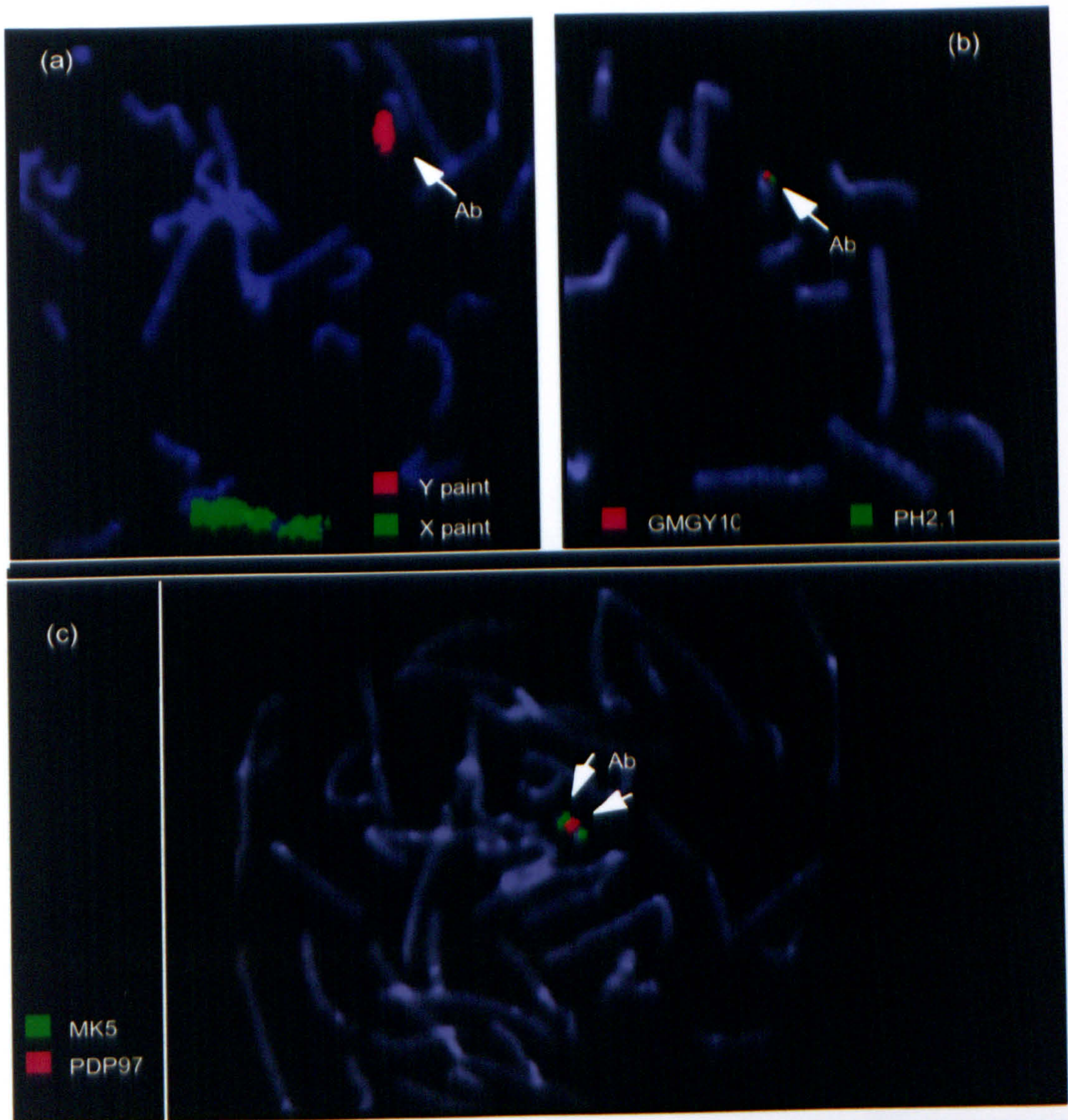


Figure 3.29: FISH investigation of a patient with a 46,XY/46,X,+mar karyotype. (a) Dual FISH analysis using X and Y paints on patient's metaphase spreads showed the abnormal chromosome was covered with Y paint (arrow). (b) Hybridisation of GMGY10 (Yp11) and PH2.1 (Yq12) probes showed one copy of both probes on the abnormal Y chromosome. (c) represents FISH study using MK5 (Yq11.2) and PDP97 (centromeric) probes. One copy of the MK5 locus was detected on each arm of the abnormal chromosome (arrows). Ab = abnormal.

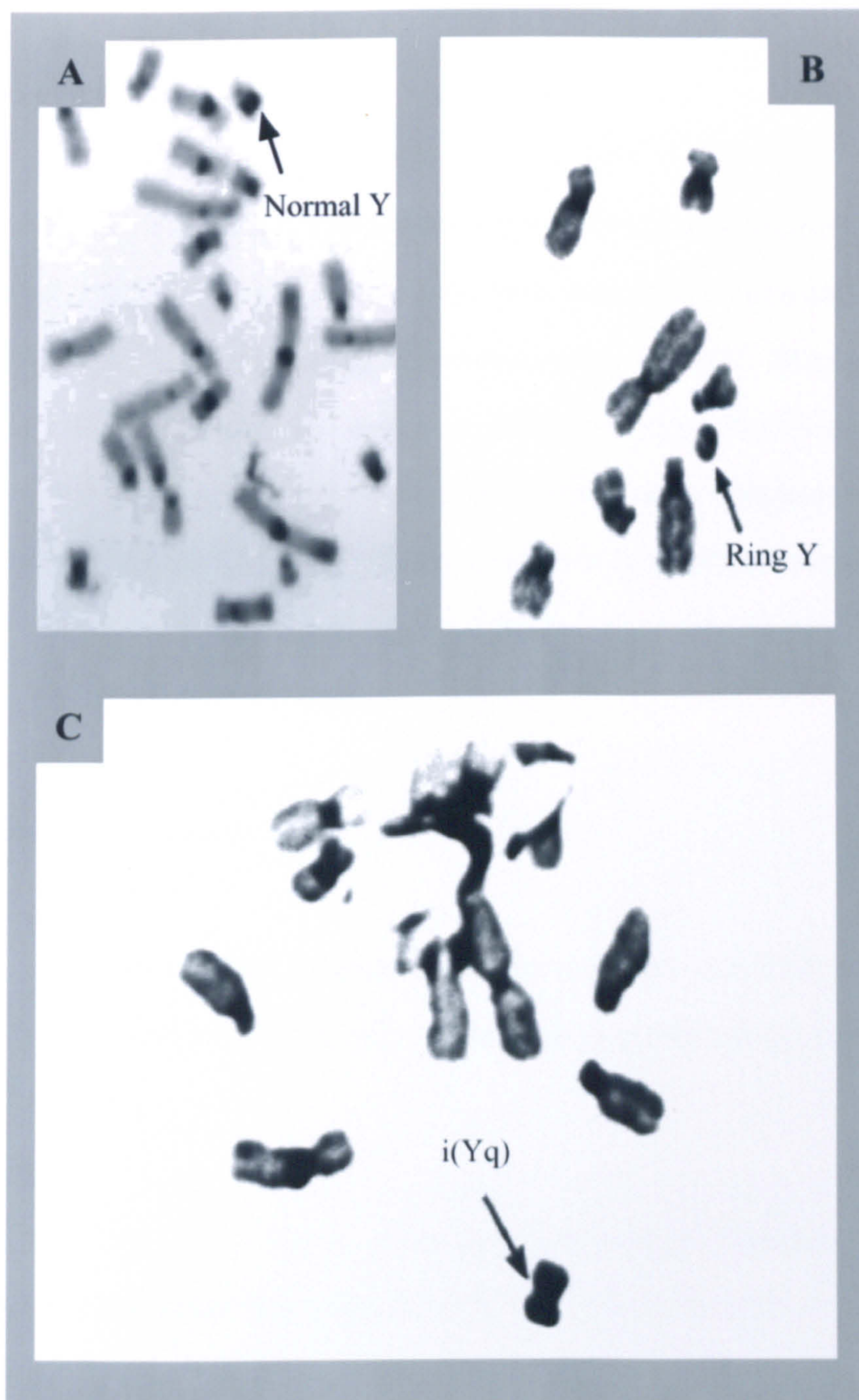


Figure 3.30: C-banded partial metaphase spreads of cytogenetically reinvestigated case # 23 showing Y chromosome abnormalities. (A) Normal Y chromosome. Two partial metaphase spreads with ring Y (B) and with i(Yq) (C).

3.1.2 X-inactivation study

In addition to the FISH studies, replication status of the ring chromosomes in 7 patients (cases 1-7) out of the previously described 9 cases and in one case with 46,XXp+ were attempted using a terminal pulse of BrdU (B-pulse) and Giemsa staining (RBG) according to Latt et al (1977) with some modifications. Replication studies were performed by introducing BrdU (39µg/ml) to lymphocyte culture 7 hours prior to harvest (case 24). This technique results in a modified R-banding pattern with lightly stained late replicating regions (Figure 3.31). One hundred cells containing rings were analysed in each case.

(1) X-inactivation study of r(X) [Cases 1-7]

Variation in the presentation of the rings was noticed during analysis of the stage in which the DNA in the ring chromosome was replicating. Details of this study are as follows:

(1) W.H.

This case has a Turner phenotype with normal mentality. The cytogenetic study of her peripheral blood showed 45,X/46,X,r(X) in the ratio of 73:27%. The r(X) was found to be small. The activation status of the X chromosomes was investigated and one hundred rings were examined. The normal X chromosome was always active, and the ring X was found to be active (early-replicating) in 50% of cells where it was present (Figure 3.32).

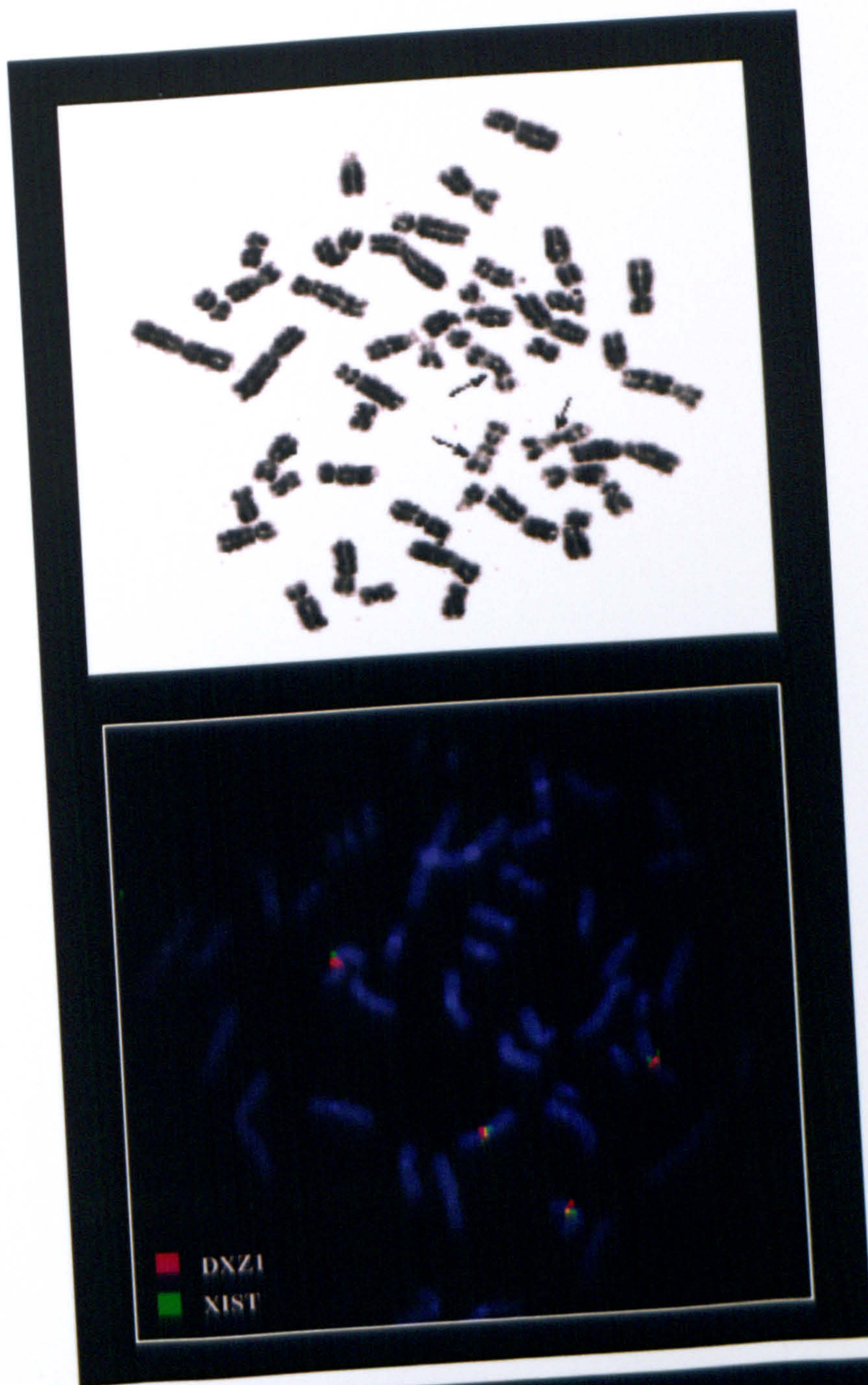


Figure 3.31: (a) Banding patterns of the early and late-replicating X chromosomes (arrows) in a 48,XXXX control case. Three X chromosomes show pale staining following BraU incorporation during the last 7 hours of culture, suggesting late-replication (inactivation). (b) FISH analysis using DXZ1 and XIST probes showing 4 copies of the X chromosome.

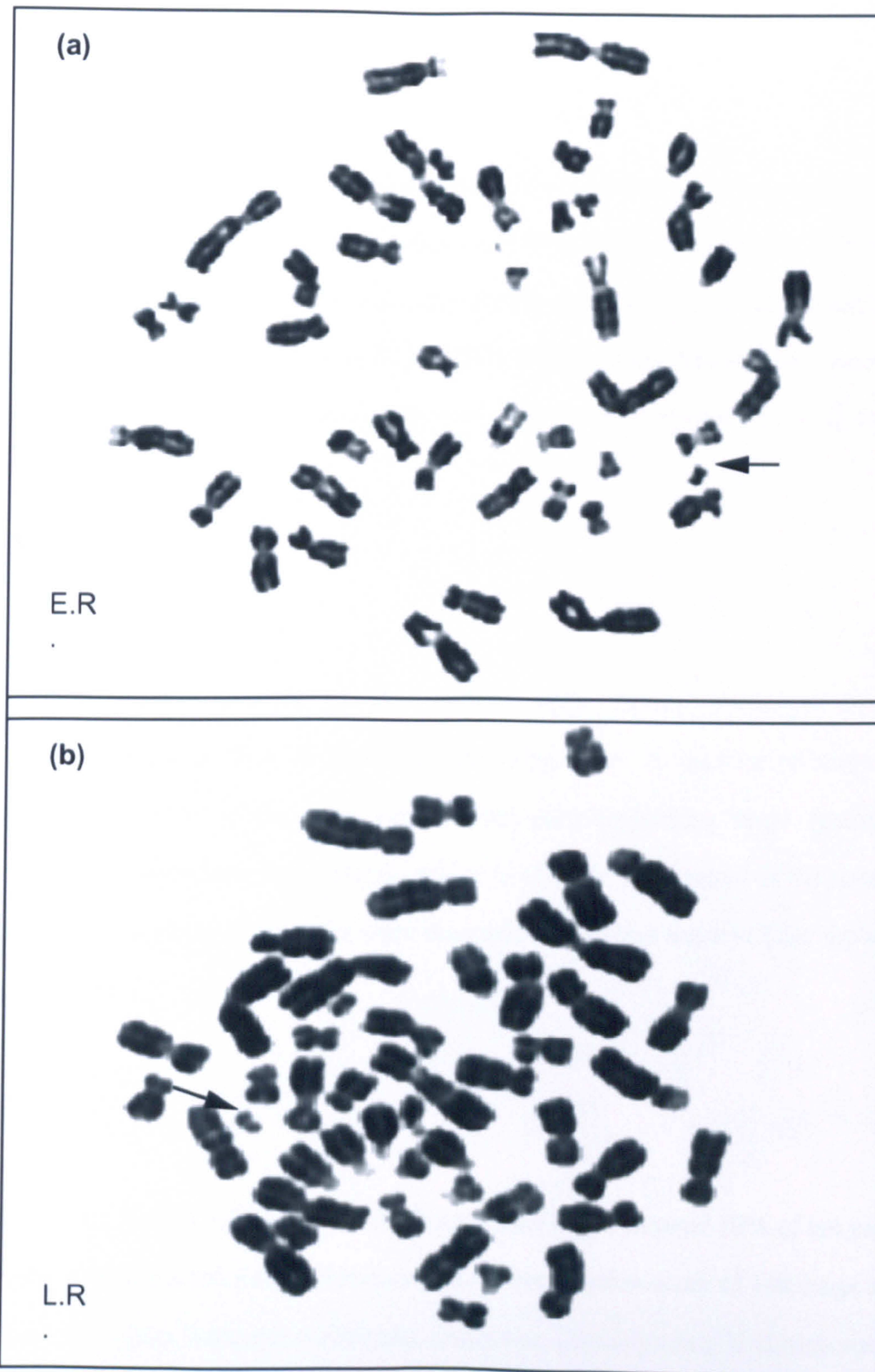


Figure 3.32: X-inactivation study in a Turner patient (W.H.) with a small ring X chromosome. (a) Shows an example of early replicating r(X). Pale staining of the ring X following BrdU incorporation during the last 7 hours of culture, suggesting late replication (inactive) (b). E.R = early replication; L.R = late replication. Arrow = r(X).

(2&3) D.C. and J.H.

Two patients with Turner phenotype who needed an extra help in school were found to have 45,X/46,X,r(X) karyotype with 28 and 20% respectively, of their cells showing small ring X chromosome on routine cytogenetic analysis. X-inactivation study of 100 r(X) showed that 50 and 46% of their rings, respectively, were active whereas their normal X chromosome was active (early-replicating) in all cell lines (Figure 3.33).

(4) F.R.

Cytogenetic analysis of this patient with Turner phenotype showed a 45,X/46,X,r(X) with 50% of medium sized ring. The X inactivation study of 100 rings revealed 14% of the rings were in the early-replicating stage (active). The normal X chromosome was always active in all cells irrespective of the state of the ring X. Two percent of her rings were dicentric and always inactive (late-replicating) (Figure 3.34).

(5) C.B.

A 45,X/46,X,r(X) patient with Turner phenotype showed 10% of her cell lines carrying medium sized ring X chromosome. X-inactivation study of 100 rings showed 88% of the rings were late-replicating (inactive). One large ring X chromosome was found and it was in the late-replicating state (Figure 3.35).

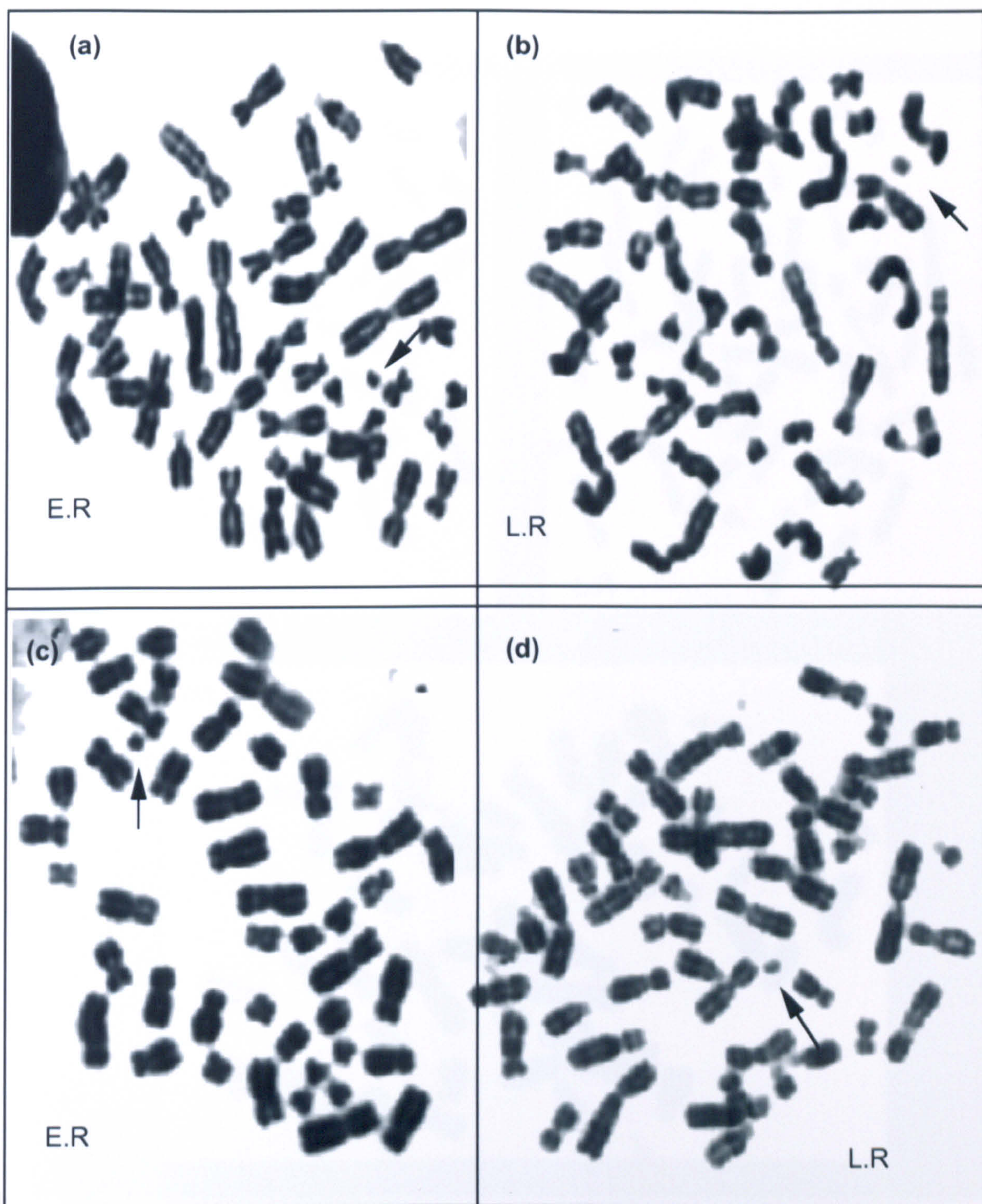


Figure 3. 33: X inactivation study in two cases with small rings (D.C. & J.H.) (a, c) showing examples of early replicating (active) ring X chromosomes detected in both cases. (b, d) Late replicating (inactive) rings were also detected in both cases (50% in D.C. and 44% in J.H.).

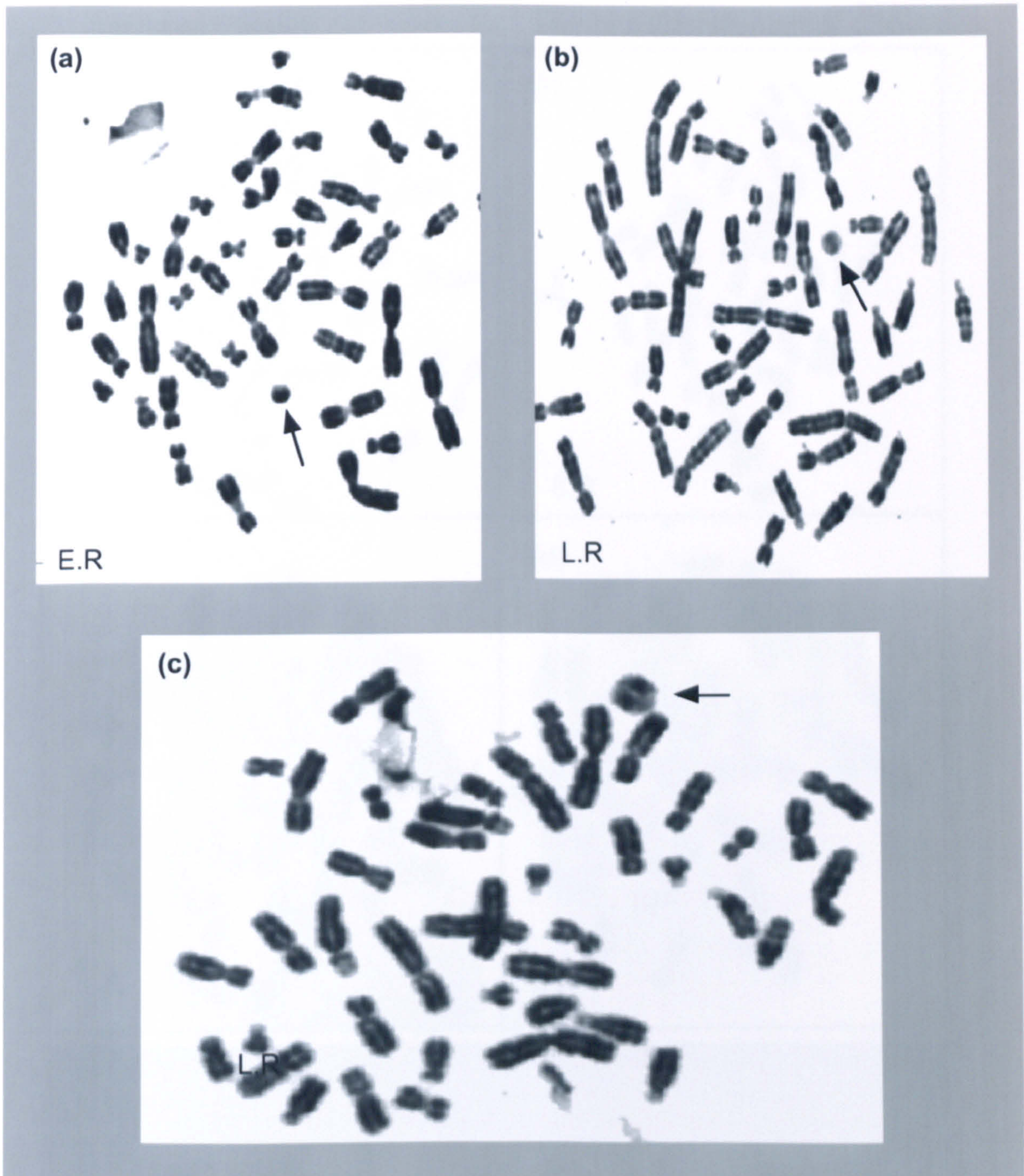


Figure 3.34: BrdU labelled replication study in a Turner Patient (F.R.) carrying a medium size ring X chromosome. (a) 14% of r(X) were observed in the early state of replication (active). (b, c) show late replicating r(X) (inactive). 2% of rings were dicentric and were inactive (c). E.R = early replication; L.R = late - replication; Arrow = r(X).

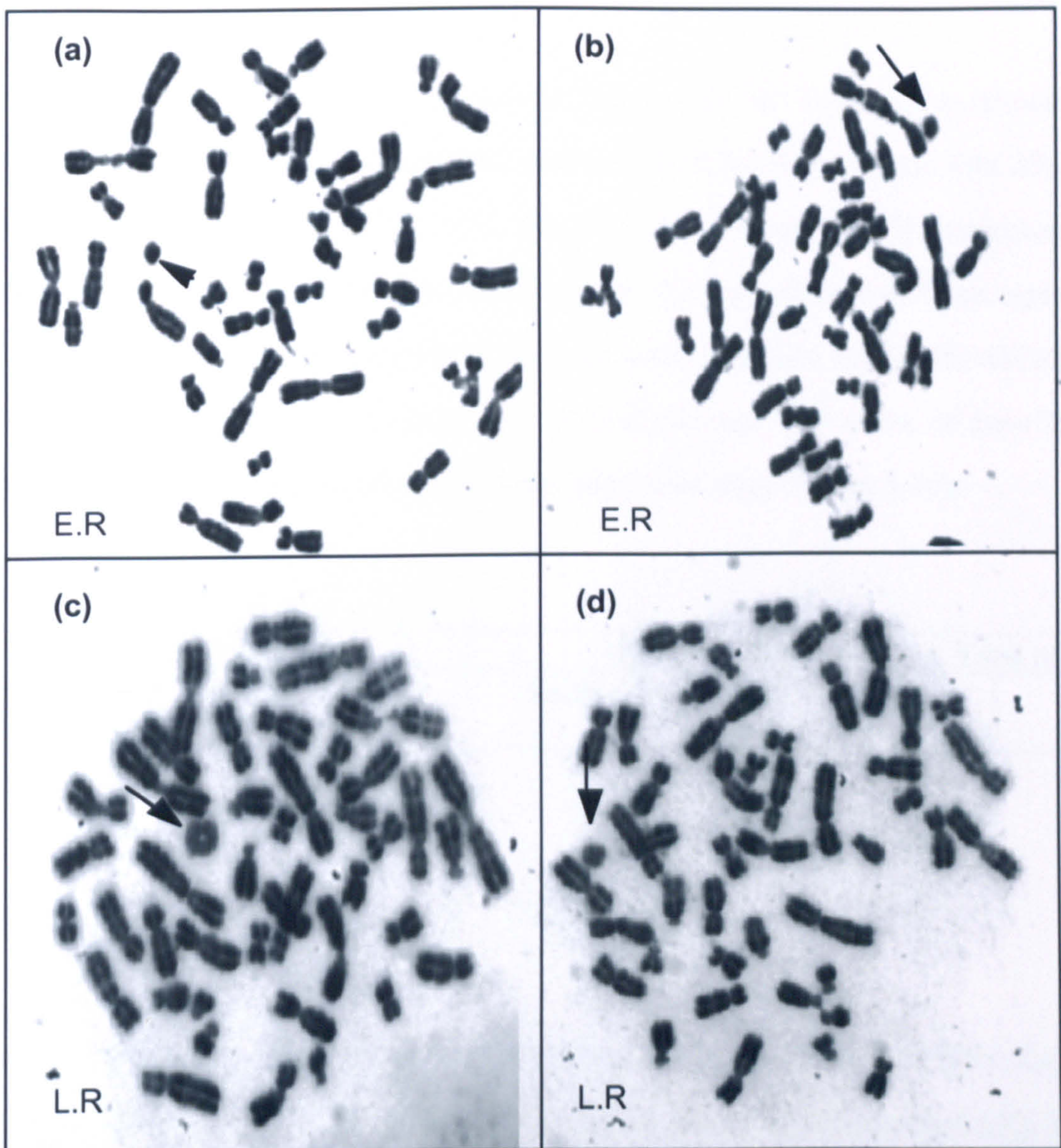


Figure 3.35: X inactivation study of a Turner syndrome case (C.B.) who is carrying a medium size ring X chromosome. (a, b) Show two medium size ring X early - replicating. One large (c) and one medium ring X showing late - replication (d). E.R. = early - replicating (active); L.R. = late - replicating (inactive).

(6) C.F.

This case had a Turner phenotype and needed an extra help in schooling. Cytogenetic analysis of mitosis cells showed a 45,X/46,X,r(X) karyotype with 20% of her cells carried medium sized r(X). The replication pattern of the X chromosomes was carried out. Critical analysis of 100 rings (X) showed three different types of rings (Table below). The majority of the rings were of medium size (87%) while 8% were small and 5% were of large size. 31% of the rings were active, of these 20% were medium , 7% were small and 4% were large sized rings (Figure 3.36).

| Active r(X) | | | Inactive r(X) | | | Total r(X) |
|-------------|--------|-------|---------------|--------|-------|------------|
| Small | Medium | Large | Small | Medium | Large | |
| 7 | 20 | 4 | 1 | 67 | 1 | 100 |

(7) K.R.

Cytogenetic study of the peripheral blood of this case showed 45,X/46,X,r(X) [71:29] karyotype with 29% of metaphase spreads carried a medium sized r(X). The replication patterns of the normal and r(X) was done and 100 cells with 46,X,r(X) were analysed. The normal X chromosome was always active. 20% of the rings were early-replicating (active) and 80% of the rings were late replicating (inactive). 2% of rings were of large size and all were active (Figure 3.37).

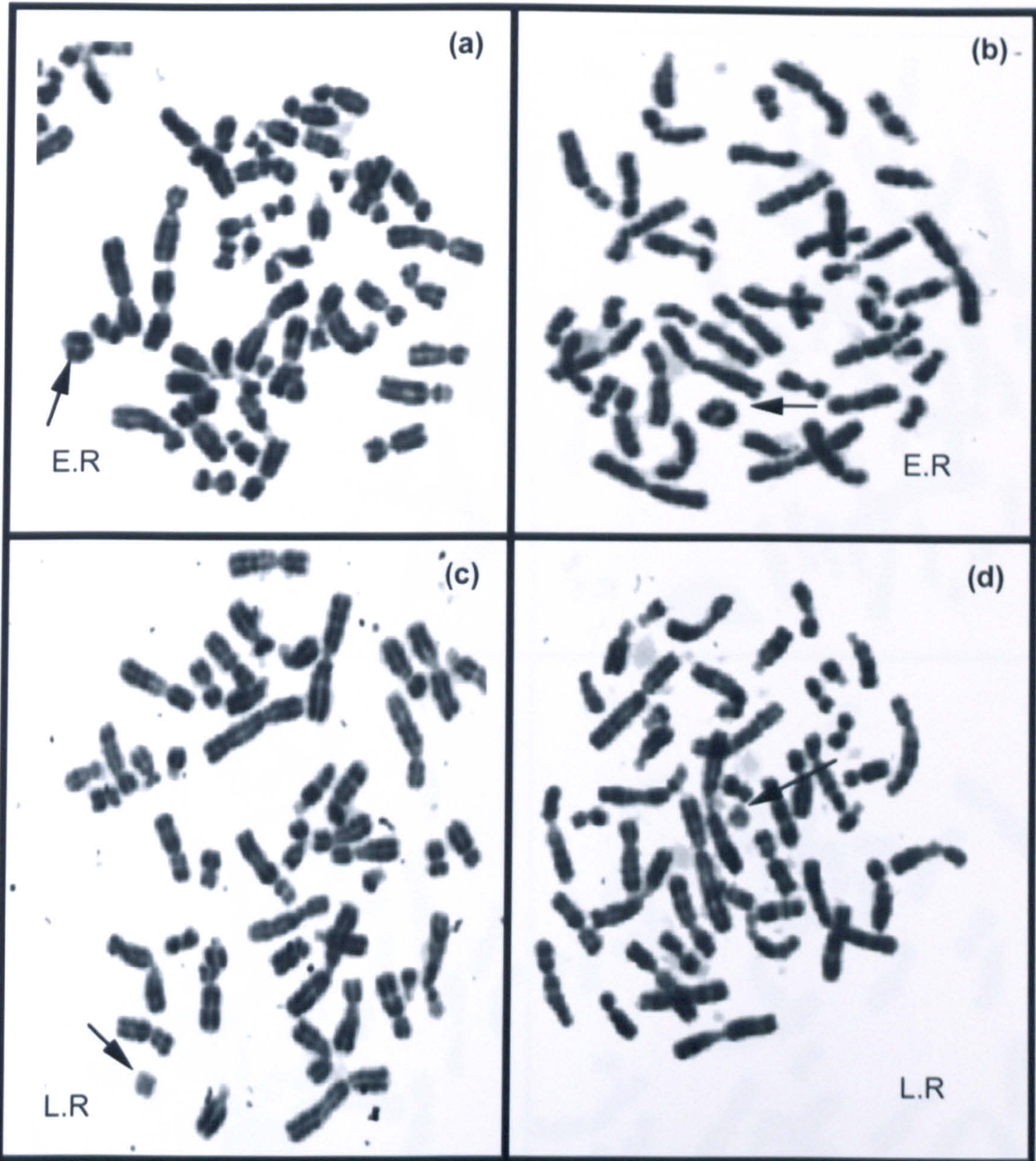


Figure 3.36: The replication pattern of X chromosomes in C.F. case. (a, b) represent two large rings early replicating (active) detected in this patient (arrowed). Figures (c, d) show two medium size rings late replicating (inactive) (arrowed). E.R = early replicating; L.R = late replicating.

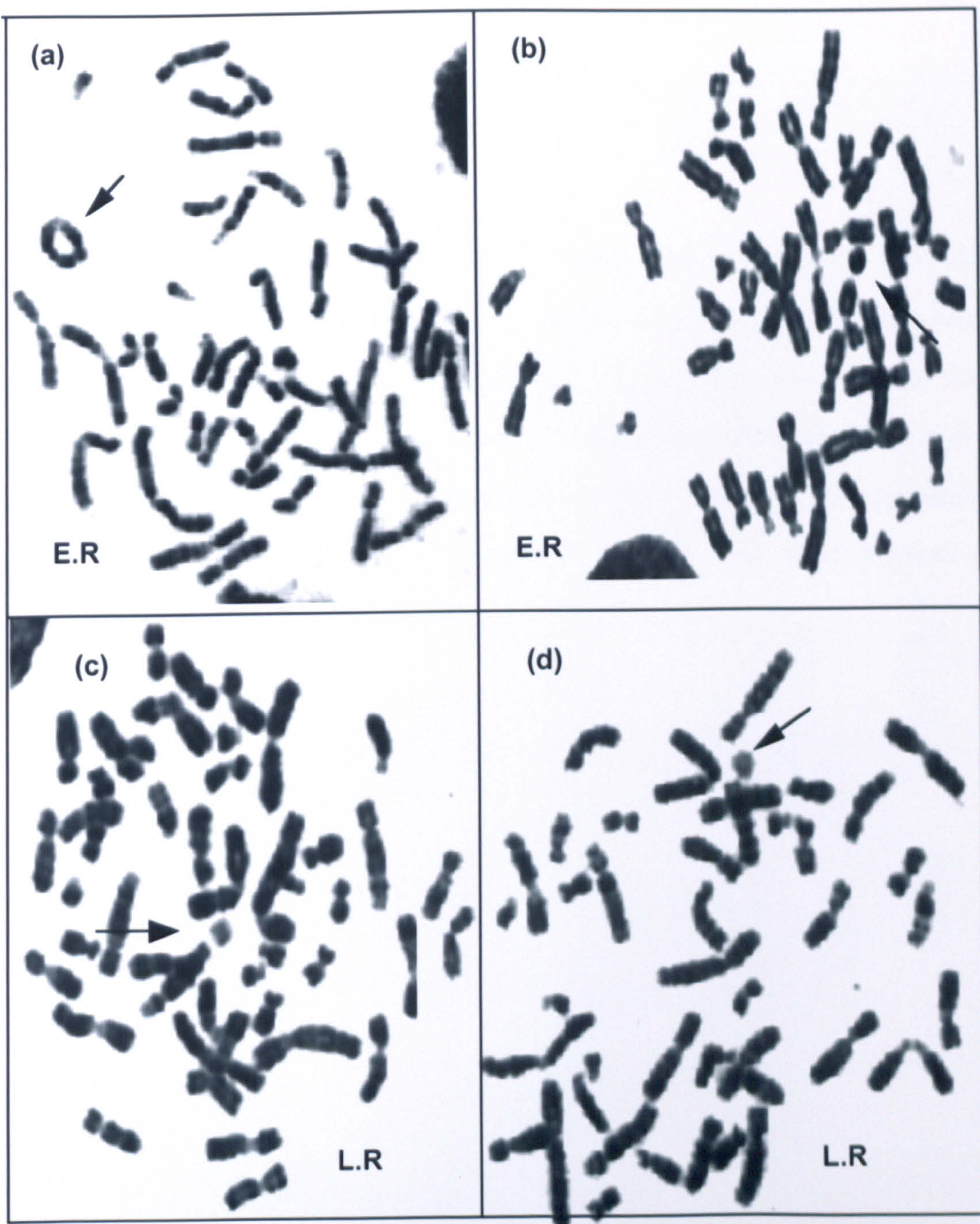


Figure 3.37: Cells from case K.R., showing early (a, b) and late (c, d) synthesis r(X) (arrows) after late pulse with BrdU. 2% of rings were found to be large in size and all were active (a). (b-d) represent medium size rings showing different pattern of r(X) replication. E.R = early - replication (active); L.R = late replication (inactive).

(2) X-inactivation study of a case with 46,XXp+

Case 20

In this case FISH investigation revealed an X/autosome [t(X;16)] translocation. The short arm of the abnormal X chromosome contained materials from chromosome 16 as previously mentioned (Figure 3.25 and 3.26). This case was also studied for the replication pattern of the abnormal and normal X chromosomes. Fifty cells were examined and all showed the abnormal X chromosome was late-replicating (inactive). The translocated segment 16q-qter on the short arm of the abnormal X showed differential replication pattern (Figure 3.38).

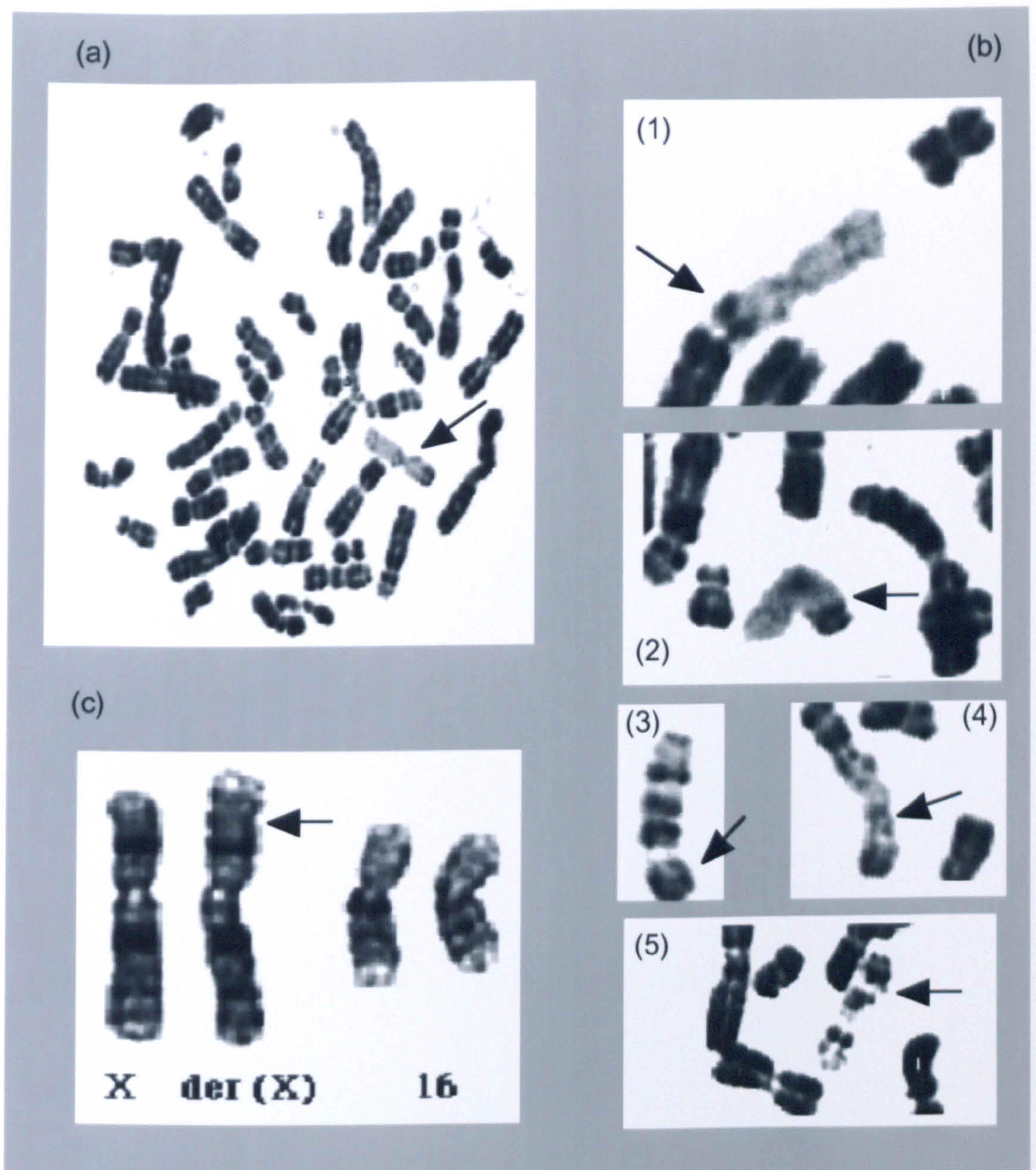


Figure 3.38: (a) The X-inactivation study of a case with 46,X,t(X:16) showing the abnormal X chromodome late replicating (arrow).(b) SHowing differential replication of the 16q on the short arm of abnormal X chromosome (arrows). (c) Partial metaphase of G-banded chromosome showing the abnormal X chromosome (arrow) and two normal chromosome 16 X and 16.

3.2 Molecular studies:

3.2.1 Androgen Receptor gene (*AR*)

The subjects evaluated in this study were five unrelated individuals with androgen insensitivity syndrome (AIS). All subjects had normal 46,XY karyotype. Three of the subjects were referred as the complete form (CAIS), previously known as testicular feminisation syndrome. Two of patients were presented at birth with genital ambiguity and diagnosed as the partial form of AIS (PAIS).

3.2.1.1 PCR amplification of the *AR* gene exons from genomic DNA

Fourteen pairs of oligonucleotide primers were used in amplification of the eight exons of the coding region of the *AR* gene (2800 bp coding sequences). Exons 2-8 were amplified from genomic DNA using flanking intronic primer sets. Exon 1 (1615 bp) was amplified using four overlapping primer sets (Figure 3.39). PCR reactions were optimised using the published conditions as starting points.

3.2.1.2 Mutation detection strategy

After PCR amplification of all the *AR* exons from 5 AIS patients, PCR products were analysed on ethidium bromide-stained 1% agarose gels to detect major sequence alterations. If no alteration was detected, PCR products were screened by the means of SSCP and heteroduplex analyses to detect point mutations and minor sequence alterations. Since nearly all reported mutations in the *AR* gene were detected in exons 2-8, direct sequencing of these exons were performed from all AIS patients to detect any possible mutation that may not be detected by SSCP or heteroduplex analyses.

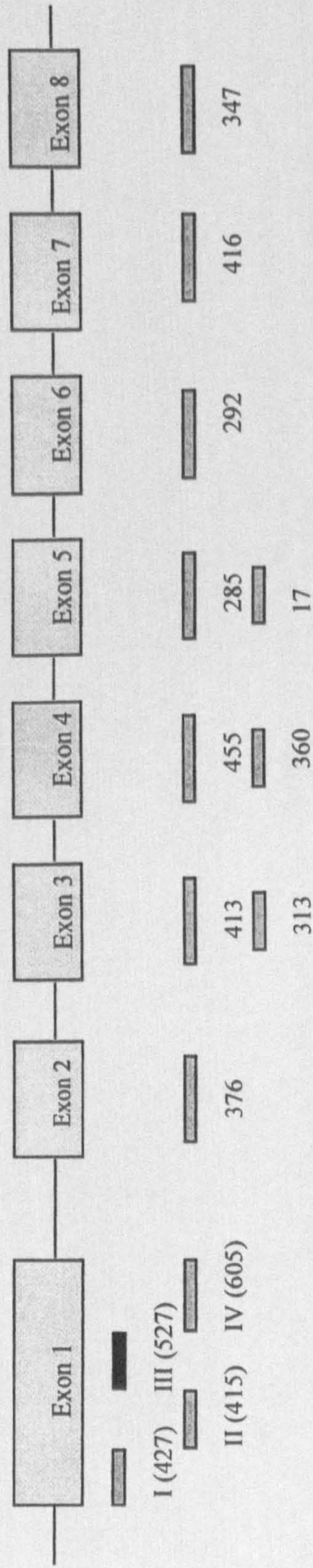


Figure 3.39: The *AR* gene with locations and sizes (base pairs) of the PCR products.

PCR amplification of all the *AR* exons from the screened patients revealed proper product sizes indicating the absence of major sequence alterations (Figure 3.40).

SSCP analysis was optimised and performed according to Orita et al (1989). Since SSCP is more sensitive when smaller PCR fragments were used, PCR products from exons 3, 5, 6 and 8 (171 to 347 bp) were used in this analysis. Denatured radiolabelled PCR products were analysed on 0.5% MDE gels with or without 5% glycerol ran at room temperature and at 4°C. A mobility shift was detected in PCR products of exon 8 from one CAIS patient when compared to other patients and a normal control (Figure 3.41).

Heteroduplex analysis was known to be simple to perform with a mutation detection rate comparable to that obtained upon the SSCP analysis. The MDE gel matrix significantly improved resolution of conformationally different, heteroduplex DNA molecules and was used by many researchers to detect single base substitutions, deletions and insertions in variety of diseases (Keen et al, 1991; Perry et al, 1992; White et al, 1992). The effective range for comparison of DNA containing single base pair mismatches is approximately 200-600 bp. Heteroduplex analysis of PCR products of the *AR* gene exons 1, 2, 4 and 7 (from 415-605 bp) from all patients was performed on 50 cm 1X MDE gels. No abnormal migration was detected in any of them. In all experiments a positive heteroduplex control sample were included (Figure 3.42).

To confirm the nature of the mobility shift detected upon SSCP analysis of the CAIS patient direct sequencing of the asymmetric PCR products from *AR* gene exon 8

from this patient was performed. A point mutation, 4109 G→T that causes a missense Val 889 Leu change was detected (Figure 3.43). The 4109 G→T change created an extra *AluI* enzyme restriction site which upon digestion of exon 8 PCR products (347 bp) revealed four bands of 127, 109, 64 and 47 bp respectively. Digestion of exon 8 PCR products from normal controls revealed only 3 bands of 174, 109 and 64 bp respectively (Figure 3.44). A hundred normal controls were screened using *AluI* restriction analysis of their *AR* gene exon 8 PCR products and none were found to have this mutation.

To detect any possible mutations in the *AR* gene, direct sequencing of the asymmetric PCR products obtained from exons 2-8 was performed and revealed no sequence alterations.

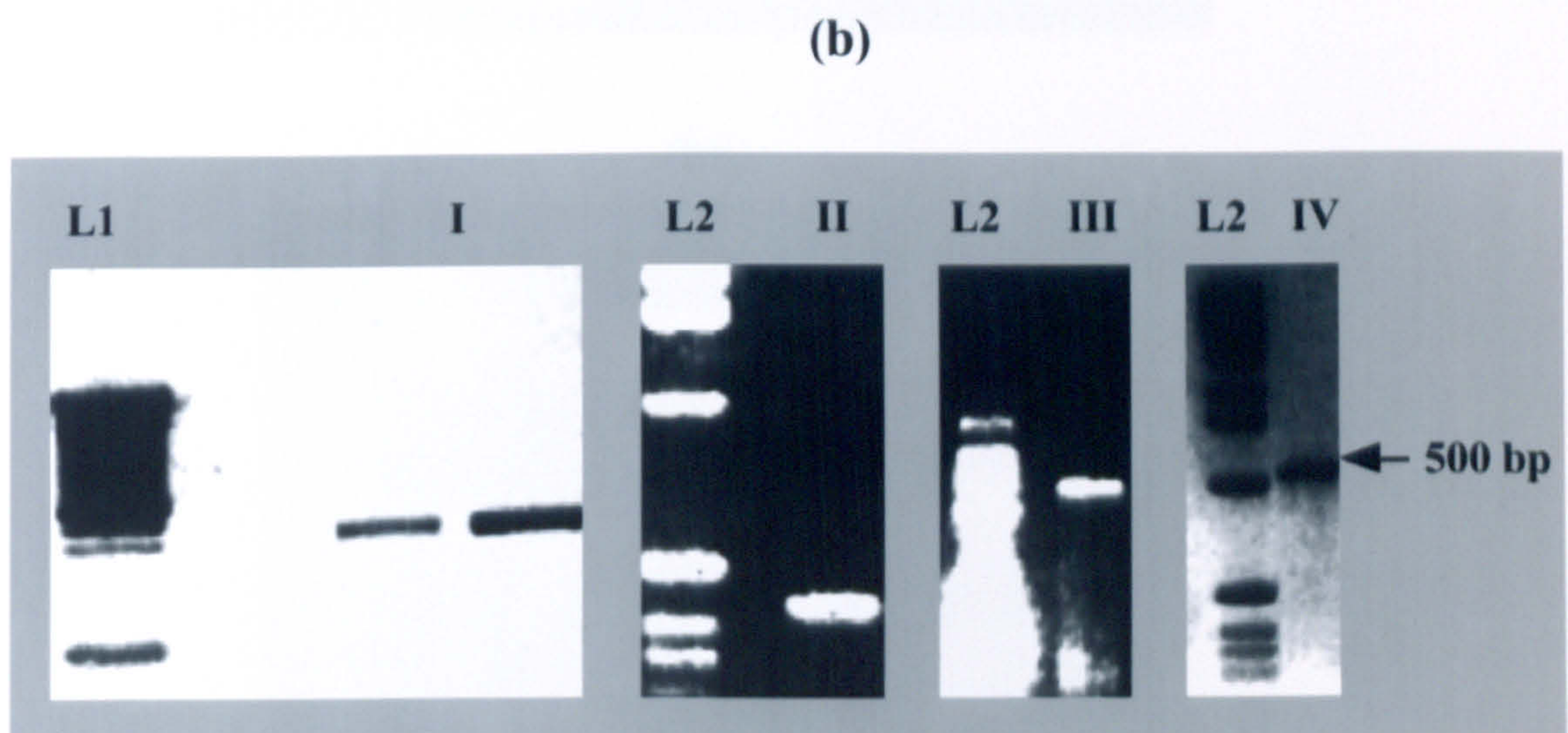
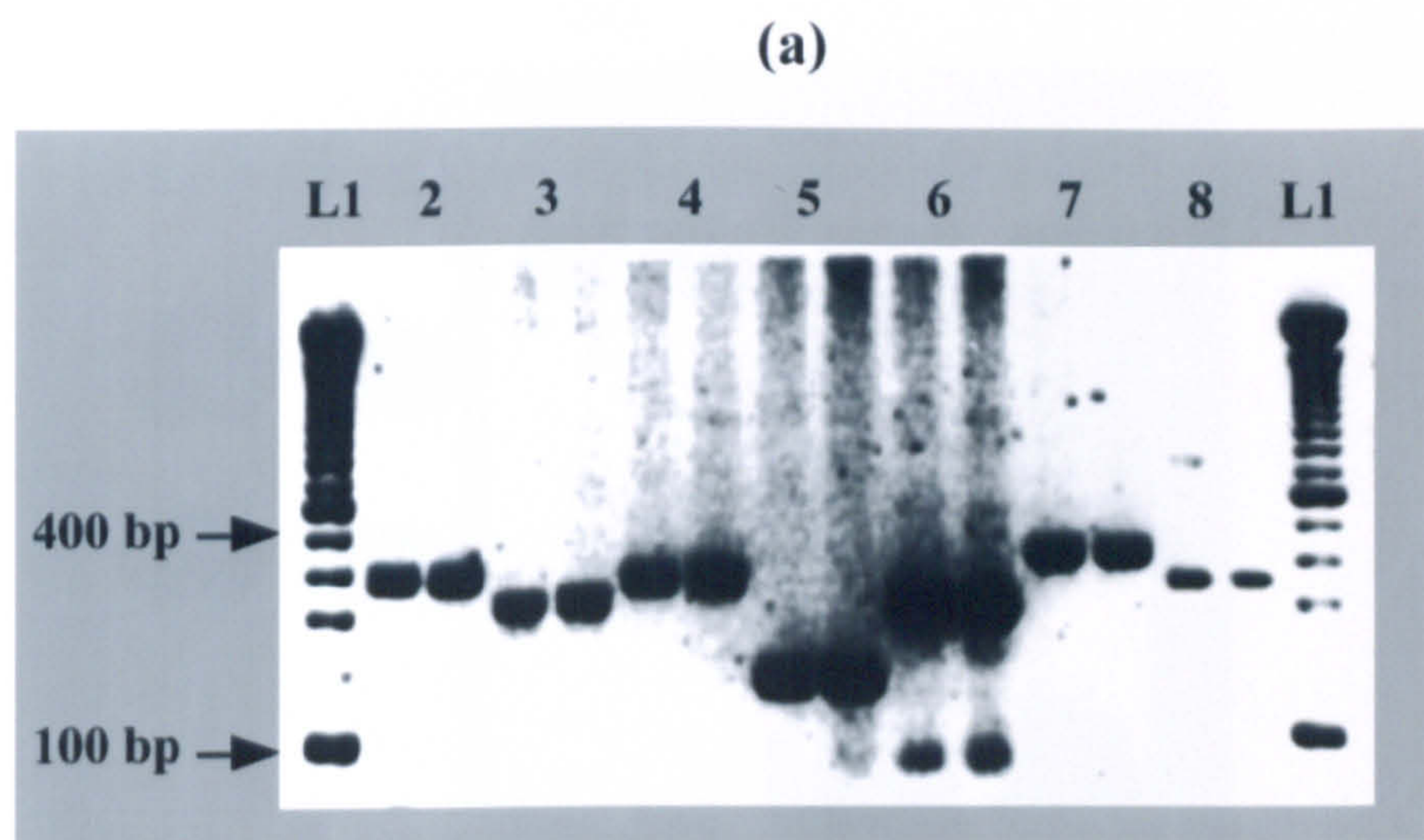


Figure 3.40: Ethidium bromide stained 1% agarose gels, electrophoresed at 100 volts for 45 minutes and photographed under UV light. **(a)** PCR products from exons 2 to 8 of the *AR* gene. **(b)** PCR products from the four segments spanning exon one of the *AR* gene. Normal sized products were obtained from all screened patients indicating the absence of deletions, insertions or structural rearrangements. L1 = 100 bp DNA ladder, L2 = 1kb DNA ladder.

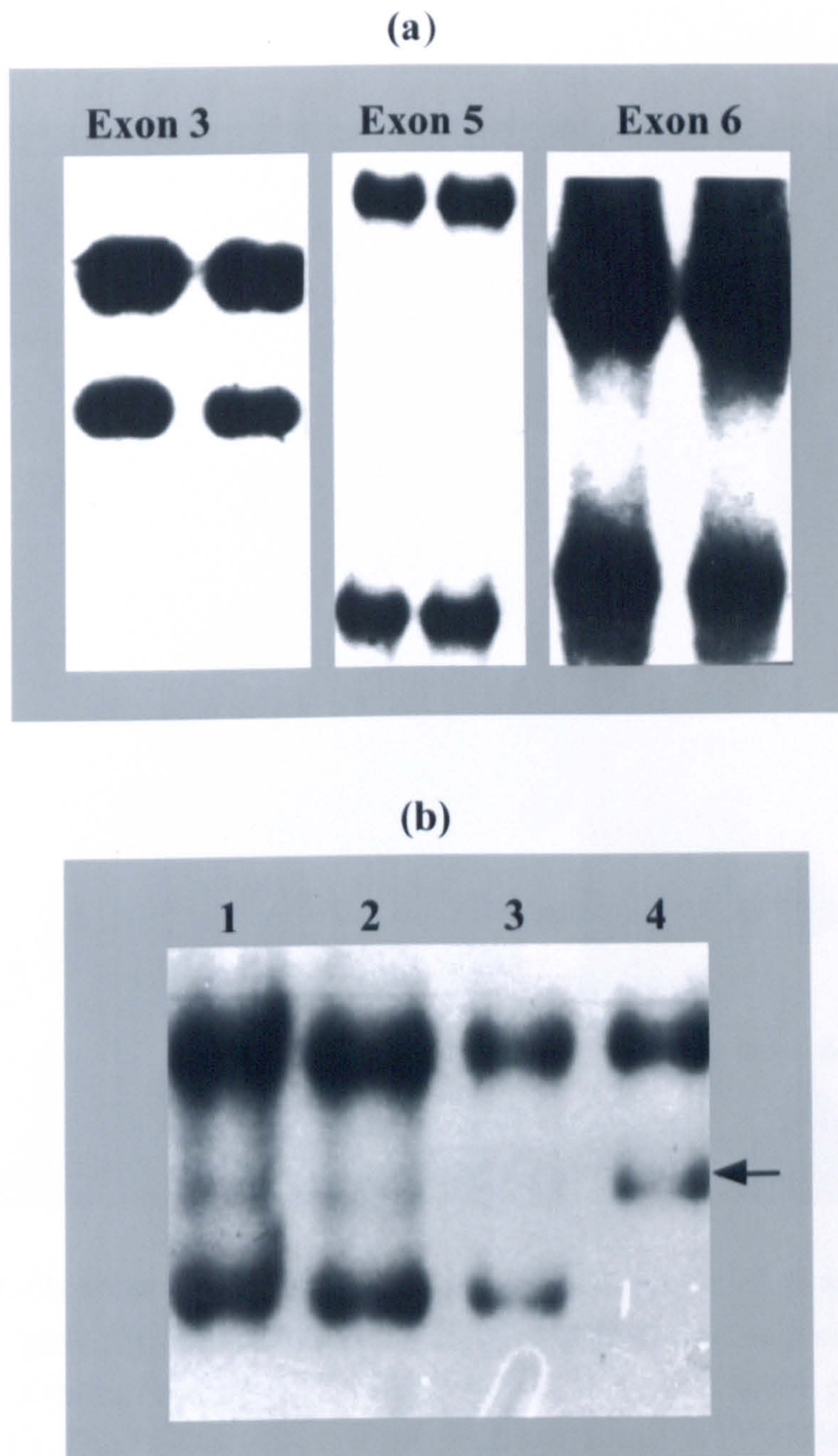


Figure 3.41: (a) Examples of radiolabelled SSCP screening of exons 3, 5 and 6 of the *AR* gene. NO abnormal band shift was detected in any of the screened patients. (b) SSCP analysis of the *AR* gene exon 8. A mobility shift (lane 4) is seen in one of the screened patients. The PCR products were electrophoresed in 0.5X MDE gel containing 5% glycerol at 5 watts for 16 hours at room temperature.

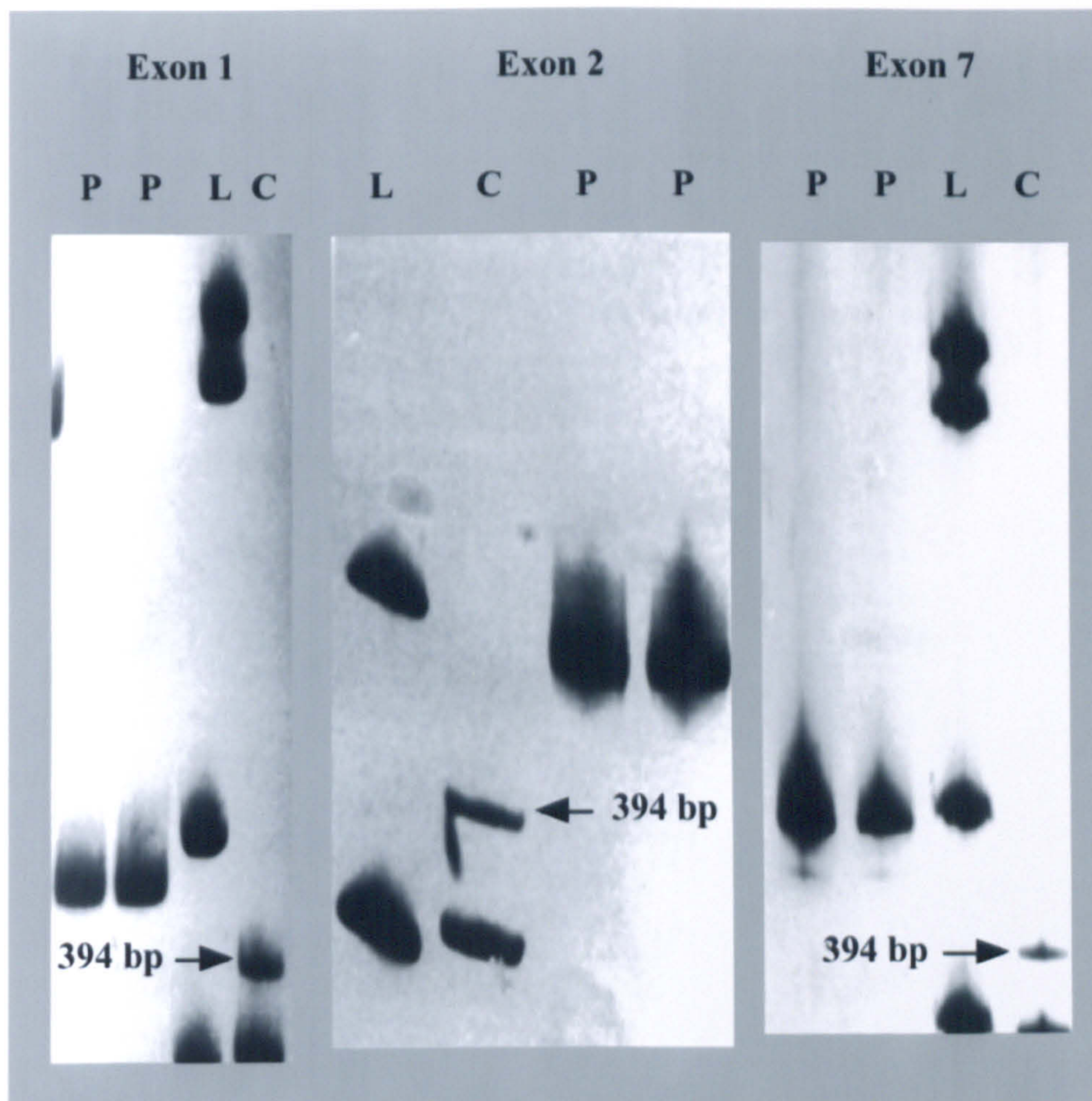


Figure 3.42: Examples of heteroduplex analysis of exons 1, 2 and 7 of the *AR* gene. No heteroduplex band were detected in any screened patients. Arrows indicate the 394 bp heteroduplex band in the positive control (C). The PCR products were electrophoresed in 1X MDE gel at 800 volts for 20 hours. The gels were stained with silver nitrate. L = 1 kb DNA ladder, C = control, P = patient.

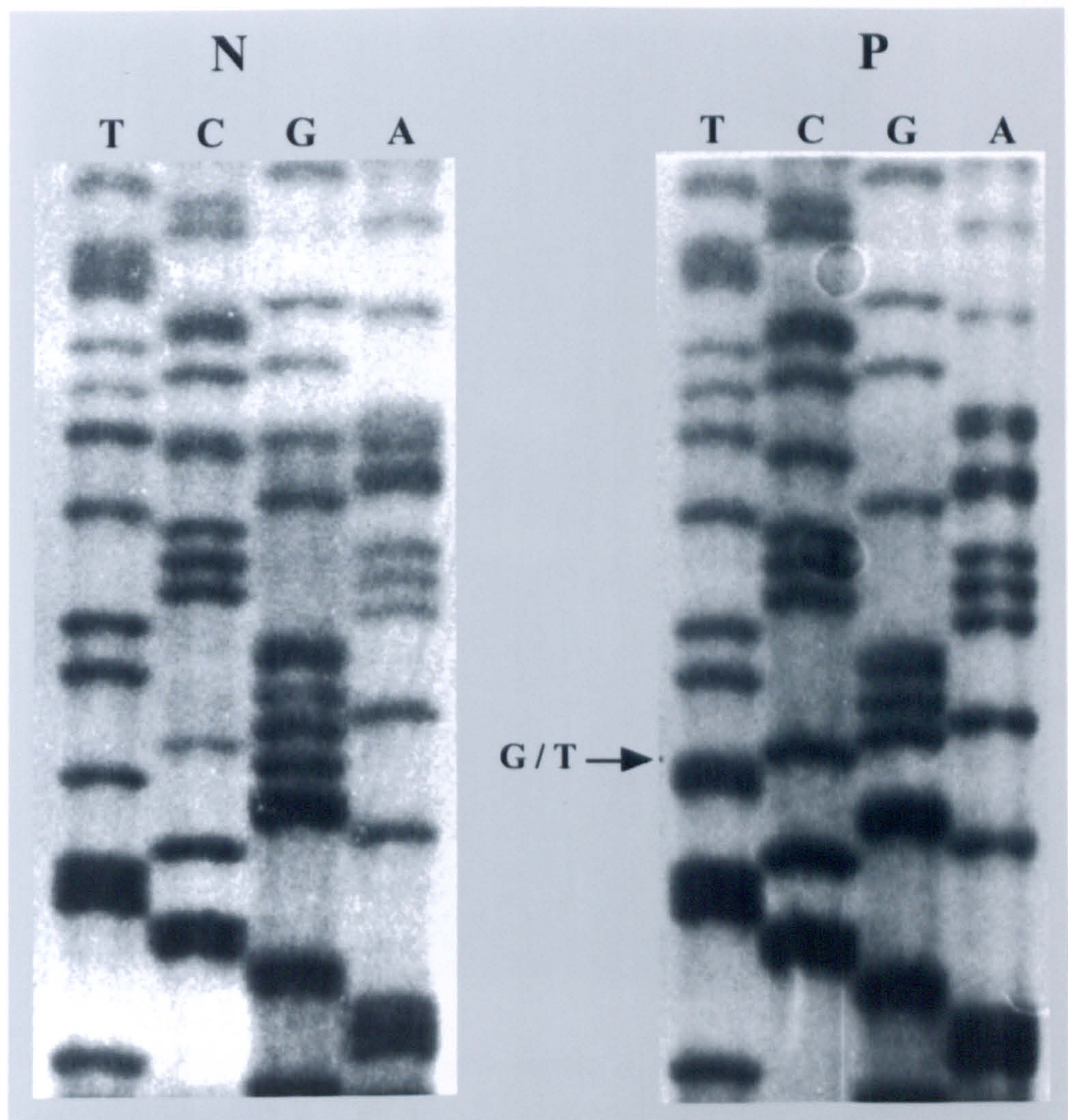


Figure 3.43: Direct sequencing of the asymmetric PCR product from exon 8 of the *AR* gene revealed 4109 G \rightarrow T mutation in the patient (P) compared to the normal control (N). The PCR products were resolved in 8% denaturing polyacrylamide gel containing 7 M urea. Electrophoresis was carried out at 50° C (~2000 volts) for 3.5 hours.

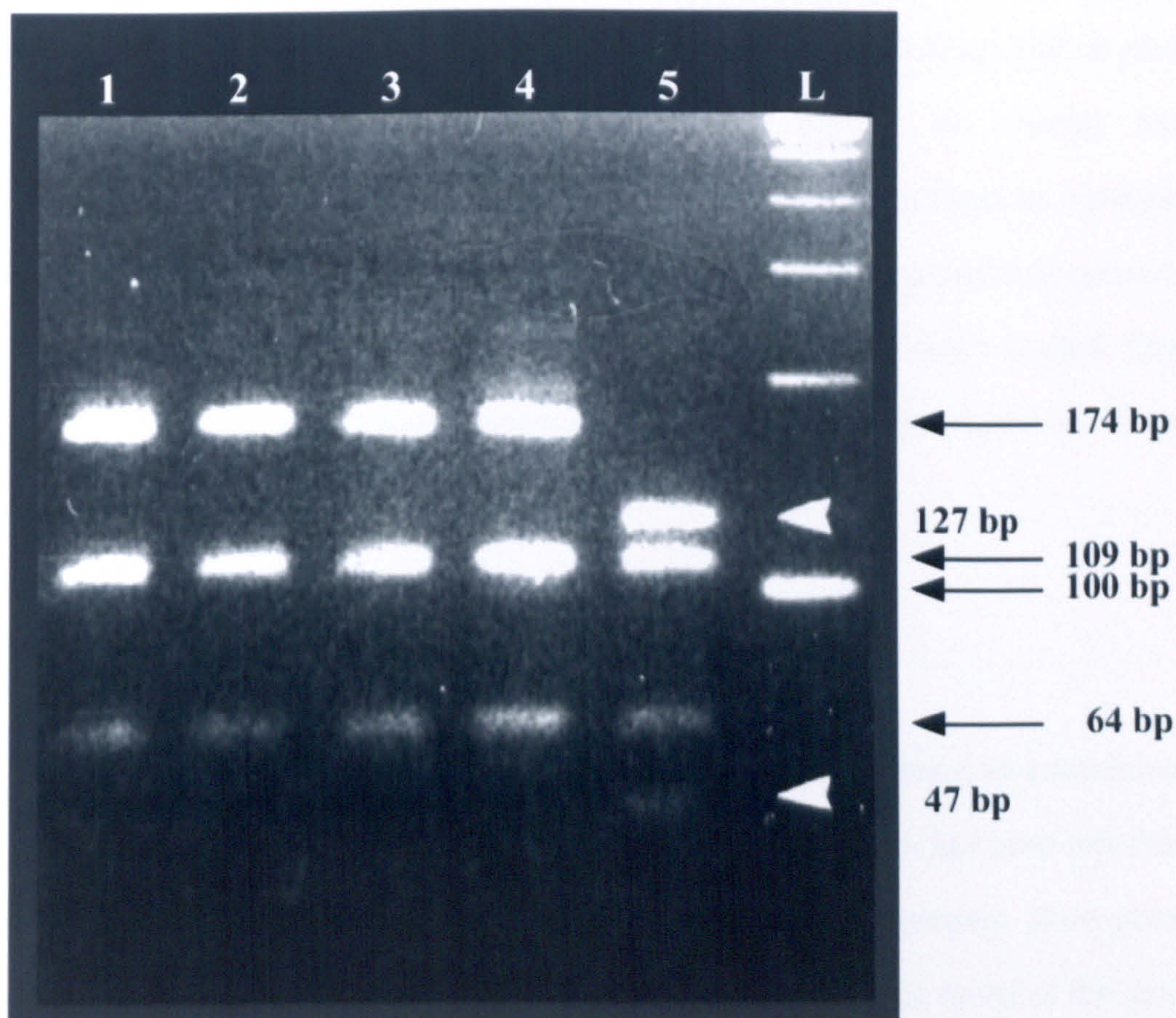


Figure 3.44: *AluI* restriction enzyme digestion of PCR products from exon 8 of the *AR* gene. Lanes 1 to 4 show normal digestion products of 174, 109 and 64 bp. Lane 5 shows the digestion products from the patient who carries the 4109 G \rightarrow T mutation. The normal 174 bp product was digested into two products of 127 and 47 bp (arrow heads) due to this mutation. The PCR digests were run on 1% agarose gel at 100 volts for 2 hours, stained with ethidium bromide and visualised under the UV light. L = 100 bp DNA ladder.

3.2.2 SRY-gene

Patients:

Subjects included in this part had abnormal sexual differentiation. Two cases presented as 46,XY females with primary amenorrhea and were diagnosed as pure gonadal dysgenesis syndrome. The ultrasound scan showed no ovaries and laparotomy study revealed streak ovaries. Two cases with ambiguous external genitalia and dysgenetic gonads were referred as 46,XY partial gonadal dysgenesis and were also investigated. Four cases with 46,XX sex reversal were studied. One was referred because of ambiguous external genitalia (undescendent testicles). Three were identified at the infertility clinic.

3.2.2.1 46,XY Females

The *SRY* gene (sex-determining region Y gene) was proposed as a candidate for the testis determining factor (TDF) (Sinclair et al., 1990) and it has been reported that approximately 10% of 46,XY females with gonadal dysgenesis show point mutations or deletions of the HMG (high mobility group) box-like motif of the gene (Jager et al., 1992). In this study, four 46,XY females with gonadal dysgenesis were analysed for the presence of major deletions, point mutations or minor sequence alterations in the *SRY* gene.

In order to detect major deletions in the TDF region, fluorescence in situ hybridisation (FISH) analysis using hocSRY (*SRY*-cDNA specific and hybridises at Yp11.2) and GMGY10 (hybridises to the TDF region at Yp11.1) probes was performed. Hybridisation study on metaphase spreads from all patients revealed positive signals of both probes at Yp11 region of the Y chromosome indicating the absence of major deletion of the *SRY* gene in the studied patients (Figure 3.45). Two cases [46,X,t(X,Y) and 46,X,i(Yq)] with cytogenetically determined deletions of the

Yp region were used as negative controls and showed no hybridisation of hocSRY or GMGY10 probes on the abnormal Y chromosomes or on any other chromosomes.

Since the *SRY* gene is a single intronless gene, that encodes 223 amino acids, its open reading frame (ORF) was PCR amplified using flanking oligonucleotide primers XES10/XES11 from the four studied patients. PCR products of expected size were obtained from all patients excluding deletions, insertions or other major sequence alterations. Moreover, PCR amplification from the two negative controls (Yp deleted patients) revealed no products from the SRY region (Figure 3.46).

With one exception (Tajima et al., 1994), all known *SRY* mutations cluster in the HMG box of the gene. Two PCR primer sets were used to amplify the whole HMG (conserved) domain in two overlapping segments (217 & 246 bp) from the four studied patients. PCR products from these segments were used in the SSCP analysis study as a first attempt to detect point mutations or minor sequence alterations. MDETM gels, with and without 5-10% glycerol, running at room temperature were used in this study. No abnormal mobility shift was detected in any of the studied patients (Figure 3.47).

As no abnormalities were detected upon the SSCP analysis, the whole open reading frame (ORF) of the *SRY* gene was PCR-amplified from all patients and screened by chemical cleavage of mismatch (CCM) analysis, yet no cleavage products were obtained from any of the screened patients (Figure 3.48).

As a last attempt to screen for mutations in the *SRY* gene of these patients, direct sequencing of the asymmetric PCR products from the whole ORF, part of the promoter sequences and part of the 3' untranslated end of the gene were performed using 6 overlapping primer pairs (Figure 3.49). No sequence abnormalities could be detected in any of the screened patients (Figure 3.50).

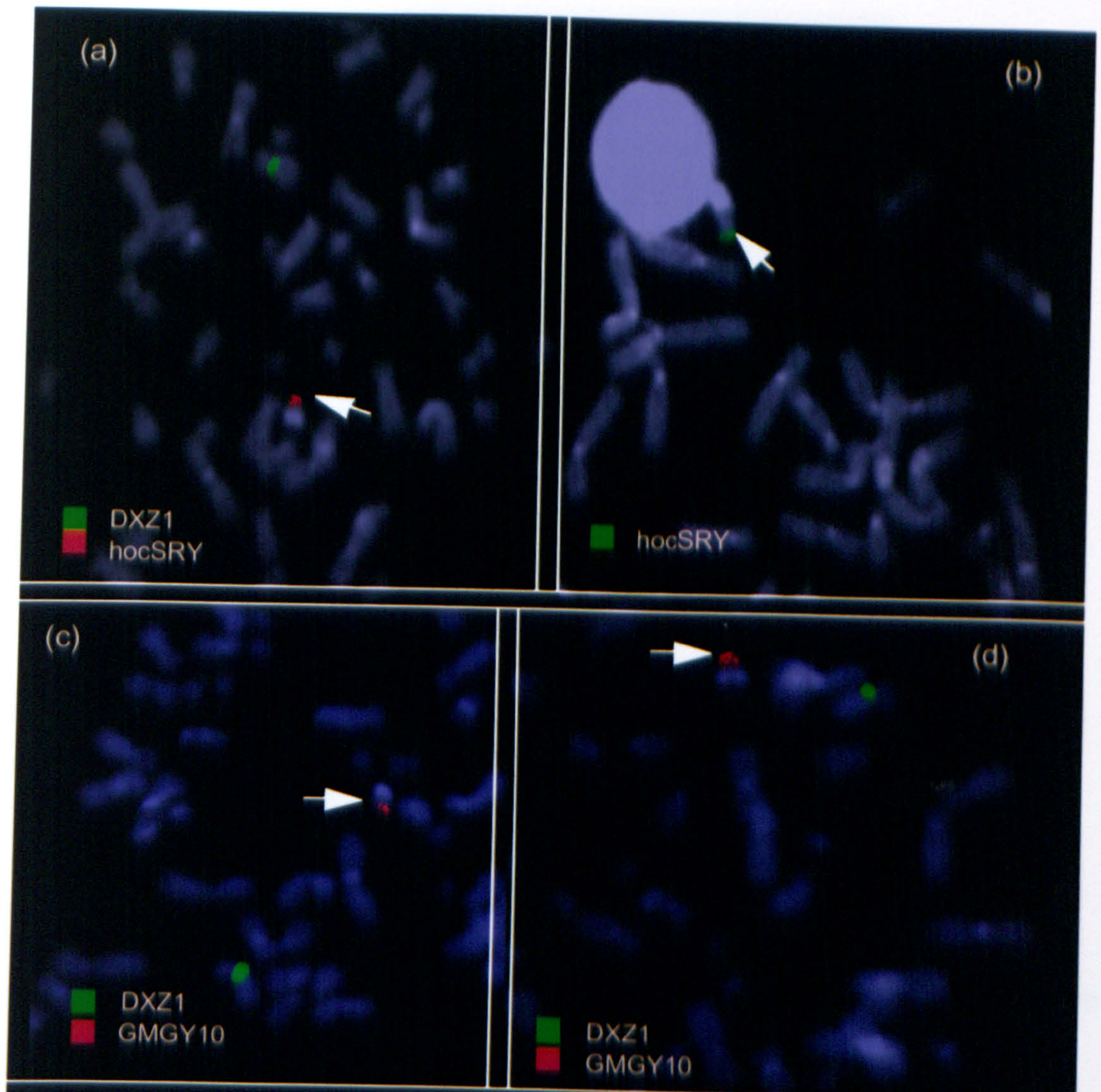


Figure 3.45: FISH study using hocSRY (a, b) and GMGY10 (c, d) probes to metaphase spreads from 4 female patients with 46,XY karyotype showing clear signals on the short arm of the Y chromosome (arrows). The green signals in a, c, and d represent hybridisation of DXZ1 to the X chromosome. The chromosomes were counter stained with DAPI.

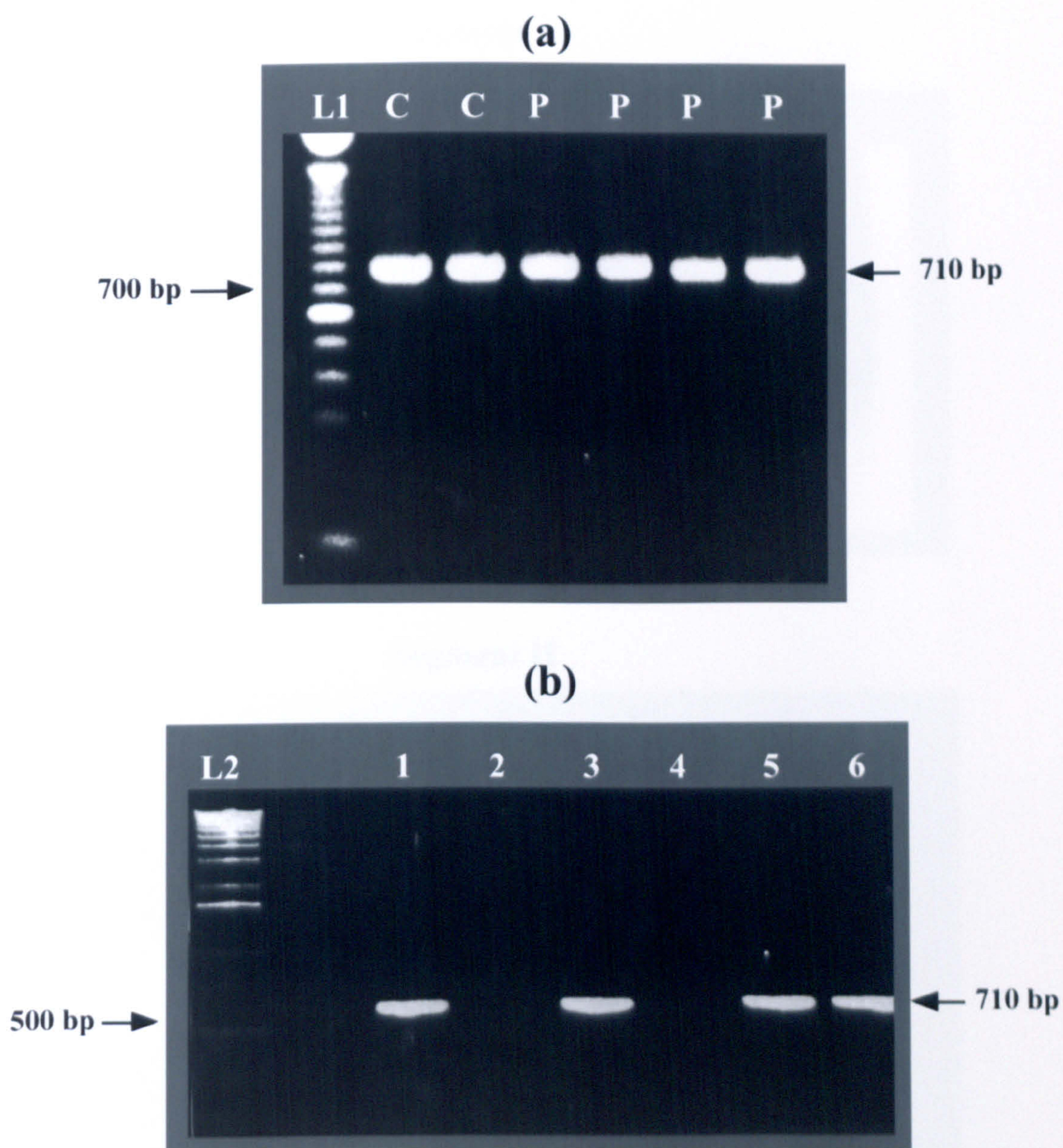
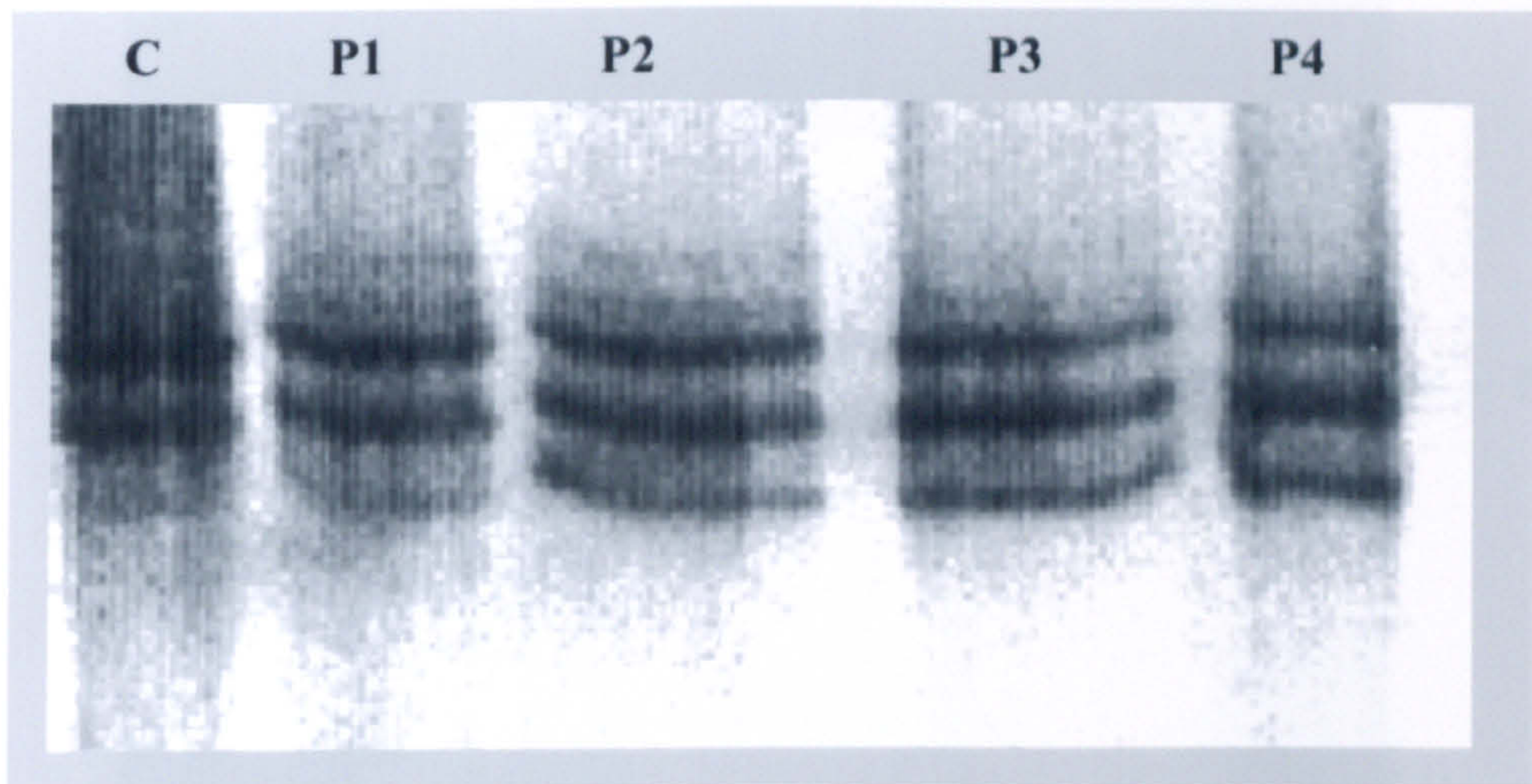


Figure 3.46: PCR amplification products (710 bp) from the open reading frame of the *SRY* gene. **(a)** No major sequence alterations could be detected in any of the screened patients. **(b)** Lanes 2 and 4 show no amplification from patients with Yp deletions (negative control). Lane 1 (normal male control) and lanes 3, 5 and 6 (three 46,XY female patients) show the 710 bp PCR products. The PCR products were run on 1% agarose gel at 100 volts for an hour, stained with ethidium bromide, visualised and photographed under UV light. C = control, P = patient, L1 = 100 bp DNA ladder, L2 = 1 kb DNA ladder.

Segment I



Segment II

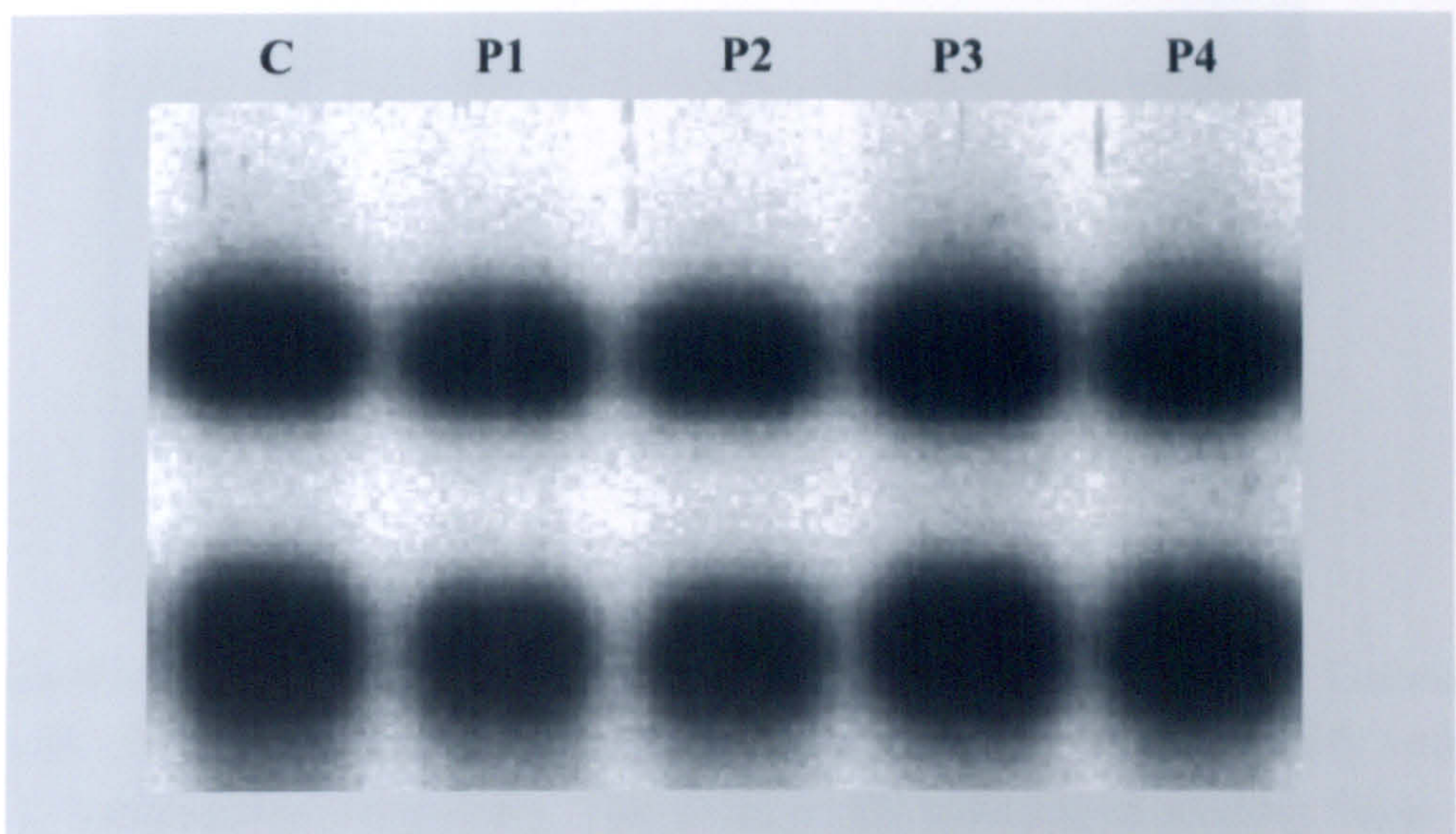


Figure 3.47: Radiolabelled SSCP analysis of the two segments that cover the HMG domain of the *SRY* gene using 0.5X MDE™ gel with 5% glycerol. No mobility shift could be detected in any of the studied patients (P). The PCR products were run at 5 watts for 16 hours at room temperature. C = control.

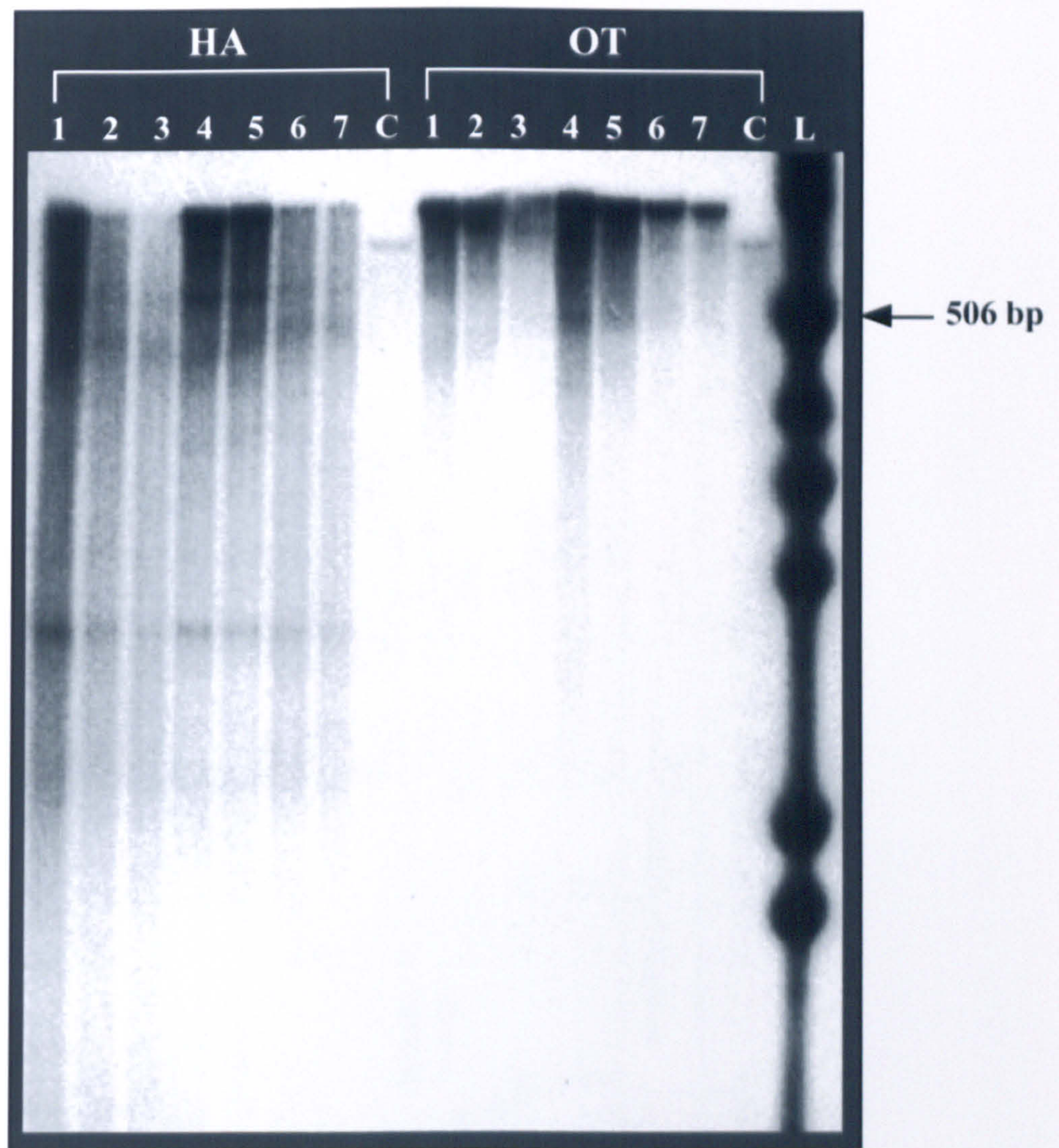


Figure 3.48: An example of chemical cleavage of mismatch (CCM). Osmium tetroxide (OT) and hydroxylamine (HA) are the chemicals used to modify either T or C mismatches respectively. The CCM of the whole open reading frame of the *SRY* gene from four female patients (1-4) with 46,XY gonadal dysgenesis and normal male controls (5-7). No cleavage products were detected upon their HA or OT modification. C = positive control; L = 1kb DNA ladder.

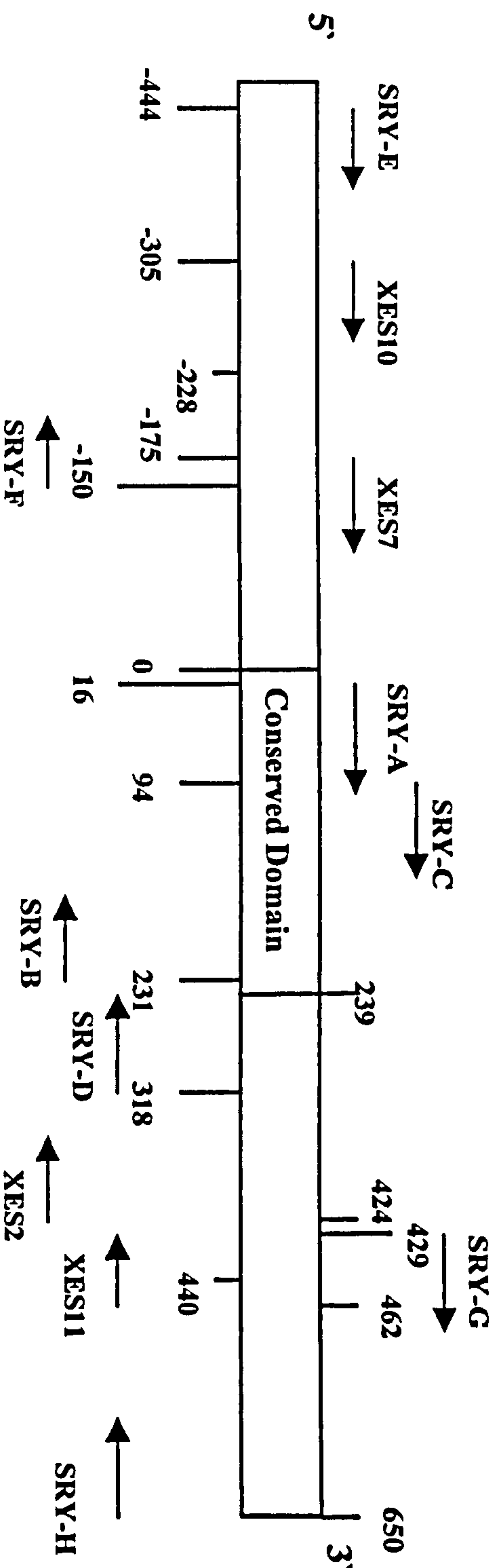


Figure 3.49: Schematic representation of the *SRY* gene indicating the positions of the primers used for PCR amplification and DNA sequencing. The start of the DNA binding domain (conserved domain) has been taken as position 0. The open reading frame starts at position -228 and ends at position 440. Primers are paired as follows: SRY-E/SRY-F, XES-10/XES-11, SRY-A/SRY-B, SRY-C/SRY-D, XES-7/XES-2, SRY-G/SRY-F.

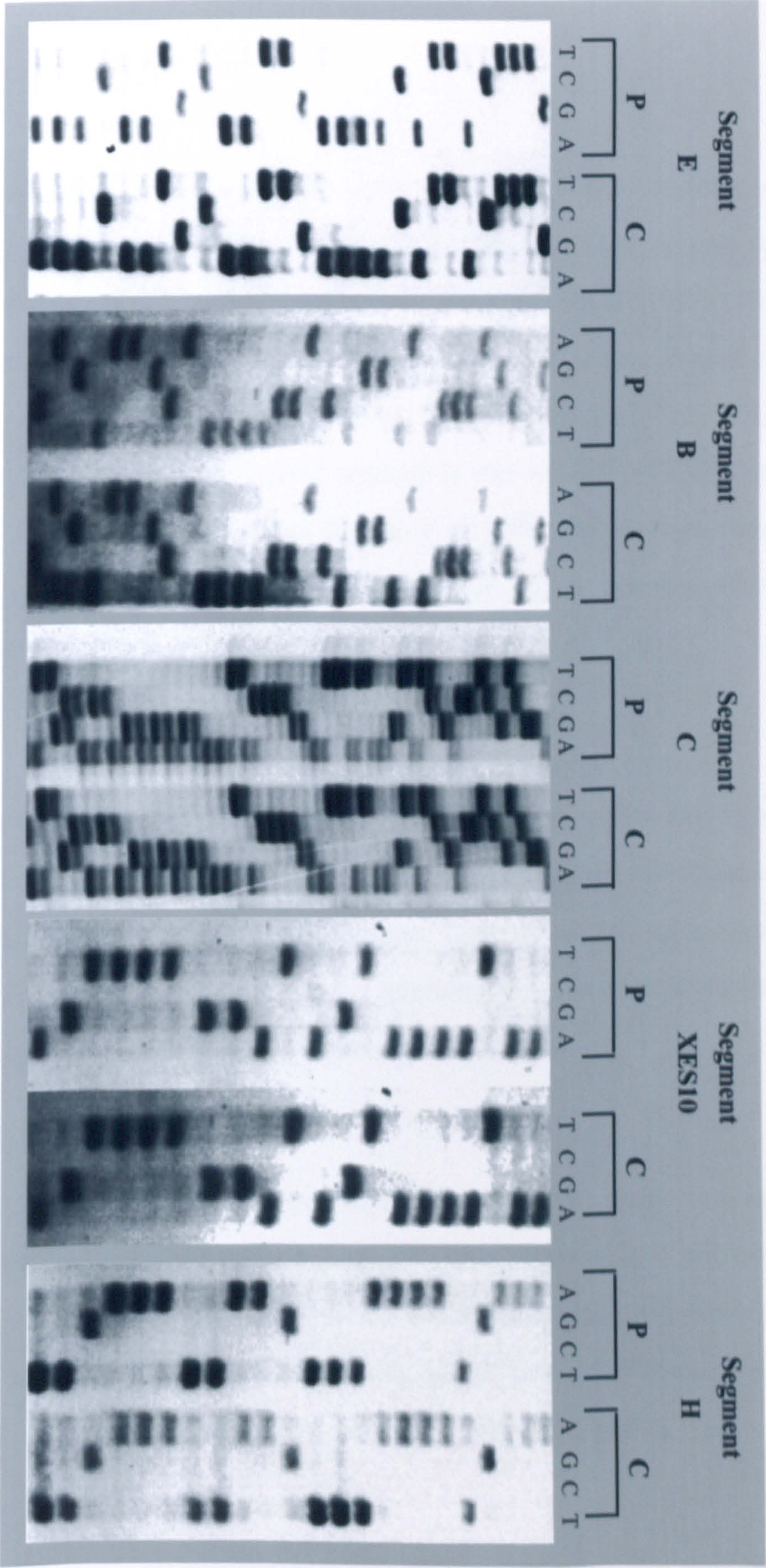


Figure 3.50: Examples of direct sequencing of the asymmetric PCR products from overlapping regions covering the *SR Y* gene ORF (segment B, C and XES10), part of its promoter (Segment E and Segment XES10) and part of the 3' untranslated region (Segment H). No sequence abnormalities were detected in any screened patients. The sequenced PCR products were resolved in 8% denaturing polyacrylamide gels containing 7 M urea. Electrophoresis were carried out at 50° C (~2000 volts) for 3 hours. P = patient, C = control.

3.2.2.2 Study of the XX males

46,XX male syndrome are one of the rarest sex chromosomal disorders in humans. In most cases the cause is due to unequal interchange between Xp and Yp termini during paternal meiosis as was proposed by Ferguson-Smith (1966). The pattern of Y-sequences present in XX males shows considerable variations. In most of the reported patients the Y segment includes almost 75% of Yp (Affara et al 1986). However, in very few cases, the translocated segment is very small (35-40 kb), but since it includes *TDF*, sex reversal occurs (Lopez et al 1995). By contrast, most 46,XX males with abnormal genitalia lack sequences for *SRY* in genomic DNA (Ferguson-Smith et al 1990).

Patients:

Four phenotypically male individuals confirmed to have 46,XX karyotype were referred for molecular investigation to exclude sex chromosome mosaicism or structural abnormalities of the X and Y chromosomes. Three out of four cases (aged between 35-48 years old) had a history of primary infertility. One 5 year old male was referred with bilateral undescendent testes (cryptorchidism).

FISH study of 46,XX males

In the present study, a 46,XX karyotype was found in the four patients with sex reversal. FISH study on metaphase spreads from all patients were carried out with PDP97 (Y-centromeric) and PHY2.1 (Yqh) probes, which detect highly repetitive Y-specific DNA sequences to exclude the possibility of XX/XY or XX/XXY mosaicism or other structural rearrangement. All patients were negative for both probes.

To detect the presence of a translocation of chromosomal material encoding *TDF* from the Y to X chromosome or autosomes, FISH analysis using hocSRY and GMGY10 probes which both hybridise to the Yp11-12 region of the Y chromosome were done. Hybridisation analysis on metaphase spreads revealed 3 cases of 46,XX males with clear signals of the hocSRY and/or GMGY10 probes on the short arm of one X chromosome. The fourth case showed no signals with either probe (Figure 3.51, Table 3.4).

PCR study

To examine the extent of transfer of Y-specific sequences in the four 46,XX males, four different Y-specific PCR primers were used. Y1/Y2 (DYZ3) primer set was used to amplify Y-centromeric sequences. No such sequences were detected by PCR in any of the 46,XX male patients compared to normal male controls confirming the FISH analysis results. PCR analysis using SRY, ZFY (zinc finger Y protein) (Yp11.2) and PAR (pseudo-autosomal region) primers that amplify DNA sequences from Yp12 region of Y-chromosome, showed amplifications of the corresponding regions in three cases of XX males. The fourth case, which showed negative FISH analysis for SRY probes, showed no PCR amplification products using the aforementioned PCR primer sets. The results are summarised in Table 3.4 and Figure 3.52.

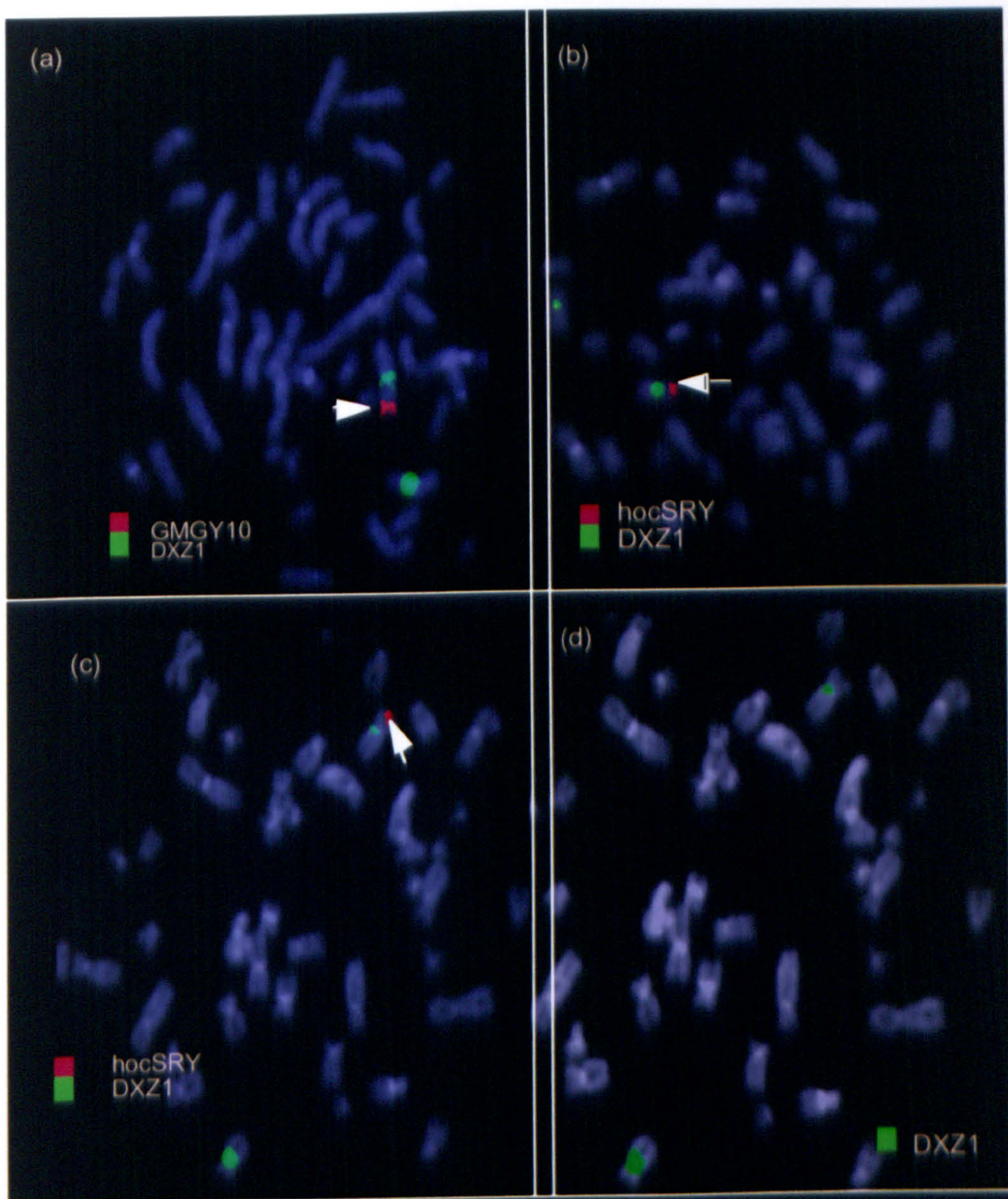


Figure 3.51: FISH analysis of 46,XX males using GMGY10 and hocSRY probes which normally hybridise to the short arm of the Y chromosome (Yp11). Three of the 46,XX males showed hybridisation signals of GMGY10 and or hocSRY probes on the short arm of one of the X chromosomes (a-c) while the 4th case showed no signals on the X chromosomes or on any other chromosomes (d). DXZ1= X-centromeric probe. The chromosomes were counter stained with DAPI.

Table 3.4: PCR amplification using Y-specific primer sets in 46,XX males and normal controls.

| Serial | Karyotype | Phenotype | Y-Cent (Y1/Y2) | SRV (XES10/XES11) | ZFY | PAR | Comment |
|--------|------------|------------------------|----------------|-------------------|------|------|---------------------------|
| 1 | 46,XX male | Ambiguous genitalia | - ve | - ve | - ve | - ve | No Y-material interchange |
| 2 | 46,XX male | Dysmorphic features | - ve | + ve | + ve | + ve | Y-material interchange |
| 3 | 46,XX male | Infertility problem | - ve | + ve | + ve | + ve | Y-material interchange |
| 4 | 46,XX male | Infertility problem | - ve | + ve | + ve | + ve | Y-material interchange |
| 5 | 46,XY | Normal male controls | + ve | + ve | + ve | + ve | |
| 6 | 46,XX | Normal female controls | - ve | - ve | - ve | - ve | |

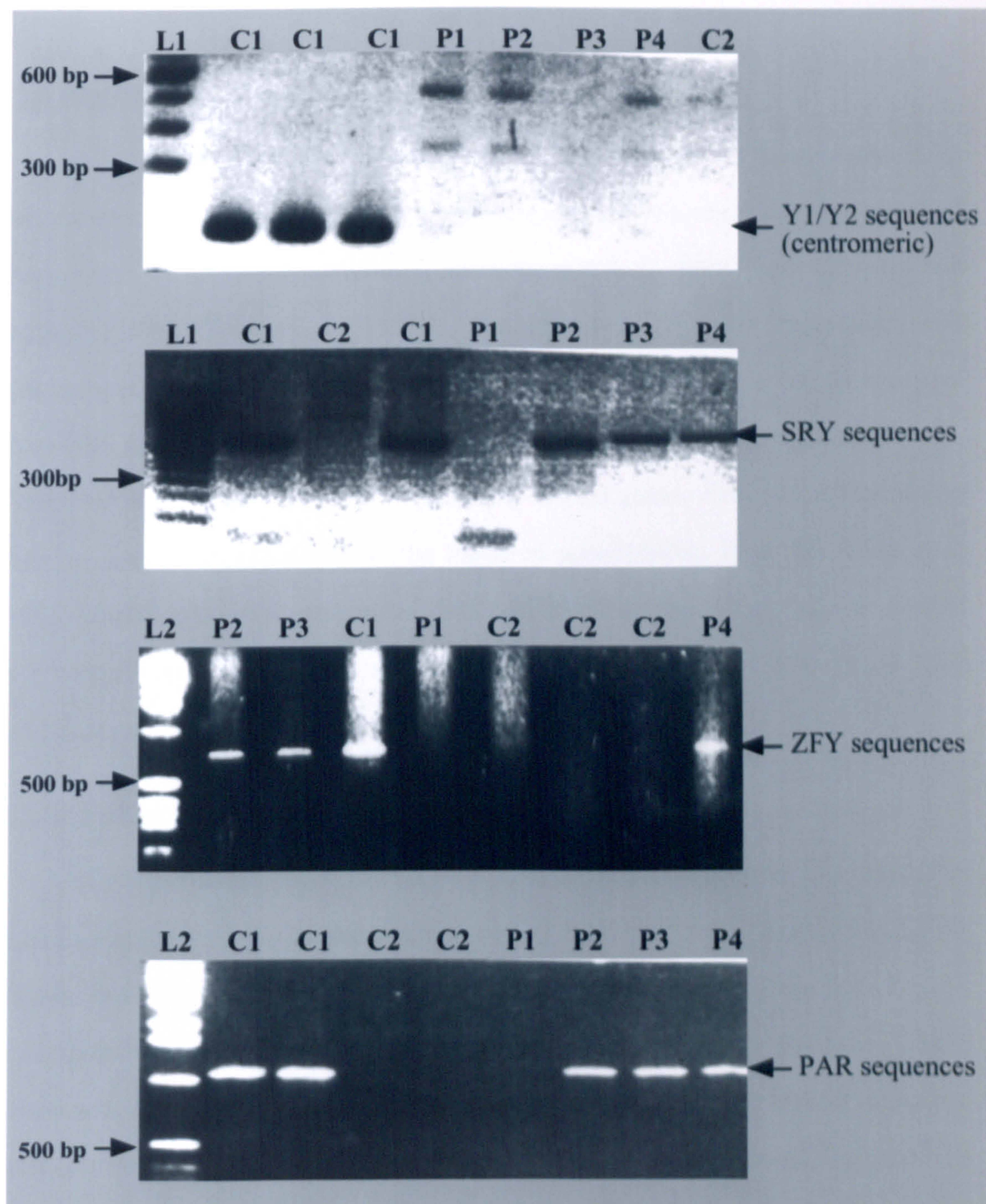


Figure 3.52: PCR amplification of DNA from the studied 46,XX males using four different Y-specific primers. Primer Y1/Y2 (170 bp) showed no amplification in all four patients (bands in P1, P2, P4 & C2 are non specific). SRY (710 bp), ZFY (735 bp) and PAR (1.1kb) primers showed amplification in three patients (P2, P3 and P4). No products were obtained from any Y-specific sequences upon PCR amplification from P1 DNA. The PCR products were run in 1% agarose gel at 100 volts for an hour, stained with ethidium bromide, visualised and photographed under UV light. P= patient; C1= normal male controls; C2= normal female control; L1= 100 bp DNA ladder; L2= 1kb DNA ladder.

3.2.3 Mutation analysis of the azoospermia factor (AZF) region

Cytogenetic analysis of anomalies in Yq11 of the human Y chromosome in sterile males with azoospermia or a severe oligozoospermia suggests the location of azoospermia factor (AZF) controlling spermatogenesis in distal Yq11 (Tiepolo and Zuffardi, 1976; Vogt et al, 1992a). The existence of genes associated with spermatogenesis on the human Y chromosome was provided by the detection of interstitial, usually submicroscopic Yq deletions in azoospermic men (Chandley et al, 1989). Ma et al. (1993) identified two closely related genes, *BM1* and *RBM2* whose absence, they proposed might be the cause of azoospermia. Recently, Reijo et al (1995) identified a novel gene, called *DAZ* (deleted in azoospermia), that was deleted in a proportion of idiopathic azoospermic men and they claimed it to be the AZF candidate gene.

3.2.3.1 Patient selection

56 infertile men aged from 22-53 years old were enrolled in this study. The clinical screening of sterile patients was started by filling in a questionnaire during clinical examination of each patient in one of the two infertility clinics from Kuwait participated in this study. Each patient was asked and examined for potential gonadal abnormalities, testis volume, varicoceles, prostatic abnormalities, chronic illnesses, previous genital injury, infection or orchitis, toxic environmental exposure or urological surgical procedures. Twelve patients had undergone testicular biopsy. Details of their spermatogenetic activity and histopathological findings were known.

Urological investigations did not reveal any kind of physical obstruction except in one case, which was then excluded from the study. One case with varicocele was also excluded from the study. Quantitative analysis of the follicle-stimulating

hormone (FSH), luteinizing hormone (LH) and testosterone were done in all cases. Patients' hormone levels of testosterone were within the normal range (5.2-22.9 nmol/L). The level of the follicle stimulating (normal ranges 1.7-11 IU/L) and luteinizing (normal ranges 0.5-5 IU/L) hormones were normal or increased. Sperm analysis of all patients showed either azoospermia or severe oligozoospermia (sperm count $< 2 \times 10^6$ / ml).

All patients were then asked for analysis of their chromosomes in order to exclude the cytogenetically detectable chromosome abnormalities known to be associated with infertility. Chromosomal study using G, Q and C-banding techniques were performed for all the 56 patients. Q and C-banding techniques showed intact Y-heterochromatic region in all patients. Four patients with small testis, showed 47,XXY karyotype (Klinefelter's syndrome). As this karyotype is known to be associated with sterility these patients were excluded from the molecular analysis. One case showed 47,XYY karyotype. Although this karyotype was not known to be associated with male infertility, yet this case was excluded because of varicocele as previously mentioned. The remaining 50 cases showed normal 46,XY male karyotype. Table 3.5 summarises the clinical and cytogenetic examination of sterile men.

3.2.3.2 Screening for deletion in the Yq11 region

PCR screening: About 10% of idiopathic sterile males were found to have microdeletion in the interval six of the Yq11 (Ma et al 1993). In the present study, 50 idiopathic sterile males with 46,XY karyotype were screened. A rapid molecular screening technique was applied and optimised using both multiplex and single PCR techniques in order to detect small interstitial deletions in Yq11. Thirty pairs of primers covering this interval were used in either single or multiplex PCR reaction. Twenty-eight Y-specific STS (sequence-tagged sites) PCR primer pairs were

optimised and used in five multiplex PCR reactions (Figure 3.53). Each multiplex experiment was repeated three times in order to evaluate the sensitivity and reproducibility of any deletion in the multiplex reactions. In case of detected deletion by multiplex-PCR analysis, experiments were repeated by single PCR reaction with primer pair(s) of the deleted STS loci. Two primers specific for the *RBM* genes were also optimised and used in single PCR reactions. The multiplex PCR study detected microdeletions in only one case (case 20) out of 50 patients. Six PCR primer products were deleted in four (out of five) multiplex reactions (Figure 3.54). The deletions detected in the multiplex PCR study were further confirmed using the specific primer sets in single PCR reactions. Screening of fifty normal fertile males, using the five multiplex PCR reactions, were performed and revealed no deletion with any primer pair. False deletions upon multiplex PCR analysis were detected. PCR amplification using the specific primer set in a single reaction revealed normal amplification from that segment (Figure 3.55).

In order to find if any of the 28 STS primers used in the multiplex reaction was located in the coding sequence of the *RBM* gene(s), Southern transfer of the five multiplex PCR reactions products was performed and the membranes were then probed using both MK5 and MK29 cDNA probes. No hybridisation signals were detected in any of the filters indicating that none of them is part of the *RBM* gene coding sequences. Because of this *RBM*-specific primers were used to screen all patients' DNA for deletions. Normal PCR amplifications from the *RBM* genes were obtained from all studied cases including the case (case 20) which showed deletions upon the multiplex PCR screening (Figure 3.56).

Table 3.5: Clinical, laboratory and cytogenetic findings in 56 infertile males.

| No | Sperm Count | Hormonal Levels | | | Chromosomal Study | Testicular Examination | Testicular Histology | Notes |
|----|-------------|-----------------|------|--------------|-------------------|-------------------------|------------------------|-------|
| | | F.S.H. | L.H. | Testosterone | | | | |
| 1 | Azoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 2 | Azoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 3 | Azoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 4 | Azoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 5 | Azoospermia | ↑ | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 6 | Azoospermia | ↑ | ↑ | ↓ | 46,XY | Left Testicle Atrophy | N.D. | |
| 7 | Azoospermia | N.D. | N.D. | N.D. | 46,XY | Normal | N.D. | |
| 8 | Azoospermia | ↑ | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 9 | Azoospermia | N | N | N | 46,XY | Left Undescended Testis | Sertoli cells only | |
| 10 | Azoospermia | ↑ | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 11 | Azoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 12 | Azoospermia | N.D. | N.D. | N.D. | 46,XY | Normal | N.D. | |
| 13 | Azoospermia | ↓ | N | N.D. | 46,XY | Normal | N.D. | |
| 14 | Azoospermia | N | N | N.D. | 46,XY | Normal | Spermatogenesis arrest | |
| 15 | Azoospermia | N.D. | N.D. | N.D. | 46,XY | Normal | N.D. | |
| 16 | Azoospermia | ↑ | N | N | 46,XY | Normal | N.D. | |
| 17 | Azoospermia | ↑ | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 18 | Azoospermia | ↑ | ↑ | ↓ | 46,XY | Small Testes | N.D. | |
| 19 | Azoospermia | ↑ | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 20 | Azoospermia | N | N | N | 46,XY | Normal | Sertoli cells only | |

Table 3.5: continue

| No | Sperm Count | Hormonal Levels | | | Chromosomal Study | Testicular Examination | Testicular Histology | Notes |
|----|------------------|-----------------|------|--------------|-------------------|------------------------|------------------------|-------|
| | | F.S.H. | L.H. | Testosterone | | | | |
| 21 | Azoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 22 | Azoospermia | ↑ | N | N | 46,XY | Small Testes | N.D. | |
| 23 | Azoospermia | ↑ | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 24 | Azoospermia | ↑ | N | N.D. | 46,XY | Normal | N.D. | |
| 25 | Azoospermia | N | N | N.D | 46,XY | Normal | N.D | |
| 26 | Azoospermia | N.D | N.D | N.D | 46,XY | Normal | N.D. | |
| 27 | Azoospermia | N.D | N.D | N.D | 46,XY | Normal | N.D. | |
| 28 | S. Oligoospermia | ↑ | N | N | 46,XY | Normal | N.D. | |
| 29 | S. Oligoospermia | ↑ | ↑ | N | 46,XY | Normal | N.D. | |
| 30 | S. Oligoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 31 | S. Oligoospermia | ↑ | ↑ | N | 46,XY | Normal | N.D. | |
| 32 | S. Oligoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 33 | S. Oligoospermia | ↑ | ↑ | N.D. | 46,XY | Normal | N.D. | |
| 34 | S. Oligoospermia | ↑ | ↑ | N | 46,XY | Normal | N.D. | |
| 35 | S. Oligoospermia | N | N | N | 46,XY | Normal | N.D. | |
| 36 | S. Oligoospermia | ↑ | ↑ | N | 46,XY | Normal | N.D. | |
| 37 | S. Oligoospermia | ↑ | N | N | 46,XY | Normal | N.D. | |
| 38 | S. Oligoospermia | N.D. | N.D. | N.D. | 46,XY | Normal | N.D. | |
| 39 | S. Oligoospermia | N | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 40 | S. Oligoospermia | N | N | N | 46,XY | Normal | N.D. | |

Table3.5: continue

| No | Sperm Count | Hormonal Levels | | | Chromosomal Study | Testicular Examination | Testicular Histology | Notes |
|----|-----------------|-----------------|------|--------------|--------------------|------------------------|---|------------|
| | | F.S.H. | L.H. | Testosterone | | | | |
| 41 | S. Oligospermia | N | N | N | 46,XY | Normal | N.D. | |
| 42 | S. Oligospermia | N | N | N | 46,XY | Normal | N.D. | |
| 43 | S. Oligospermia | ↑ | N | N | 46,XY | Normal | N.D. | |
| 44 | S. Oligospermia | ↑ | N | N | 46,XY | Normal | N.D. | |
| 45 | S. Oligospermia | N.D. | N.D. | N.D. | 46,XY | Normal | N.D. | |
| 46 | S. Oligospermia | ↑ | N | N | 46,XY | Normal | N.D. | |
| 47 | S. Oligospermia | ↑ | N | N | 46,XY | Normal | Spermatogenesis arrest | |
| 48 | S. Oligospermia | ↑ | N | N | 46,XY | Normal | N.D. | *** |
| 49 | S. Oligospermia | ↑ | N | N | 46,XY | Normal | N.D. | *** |
| 50 | S. Oligospermia | N | N | N.D. | 46,XY | Normal | N.D. | |
| 51 | S. Oligospermia | ↑ | N | N | 47,XY ^Y | Normal | N.D. | Varicocele |
| 52 | Azoospermia | N | N | N.D. | 46,XY | Normal | Obstructive type; Active spermatogenesis | |
| 53 | Azoospermia | N.D. | N.D. | N.D. | 47,XXY | Small Atrophic | N.D. | |
| 54 | Azoospermia | ↑ | ↑ | N.D. | 47,XXY | Small Atrophic | N.D. | |
| 55 | Azoospermia | ↑ | ↑ | ↓ | 47,XXY | Small Atrophic | N.D. | |
| 56 | Azoospermia | N.D. | N.D. | N.D. | 47,XXY | Small | N.D. | |

N.D. = Not determined; N = Normal; ↑ = Increased; ↓ = Decreased. *** = Two brothers; S= severe
Patients from number 51-56 were exclude from the molecular study.
Normal ranges for (a) FSH : 1.7-11.0 IU/L. (b) LH : 0.5-6.0 IU/L. (c) Testosterone : 5.2-22.9 nmol/L

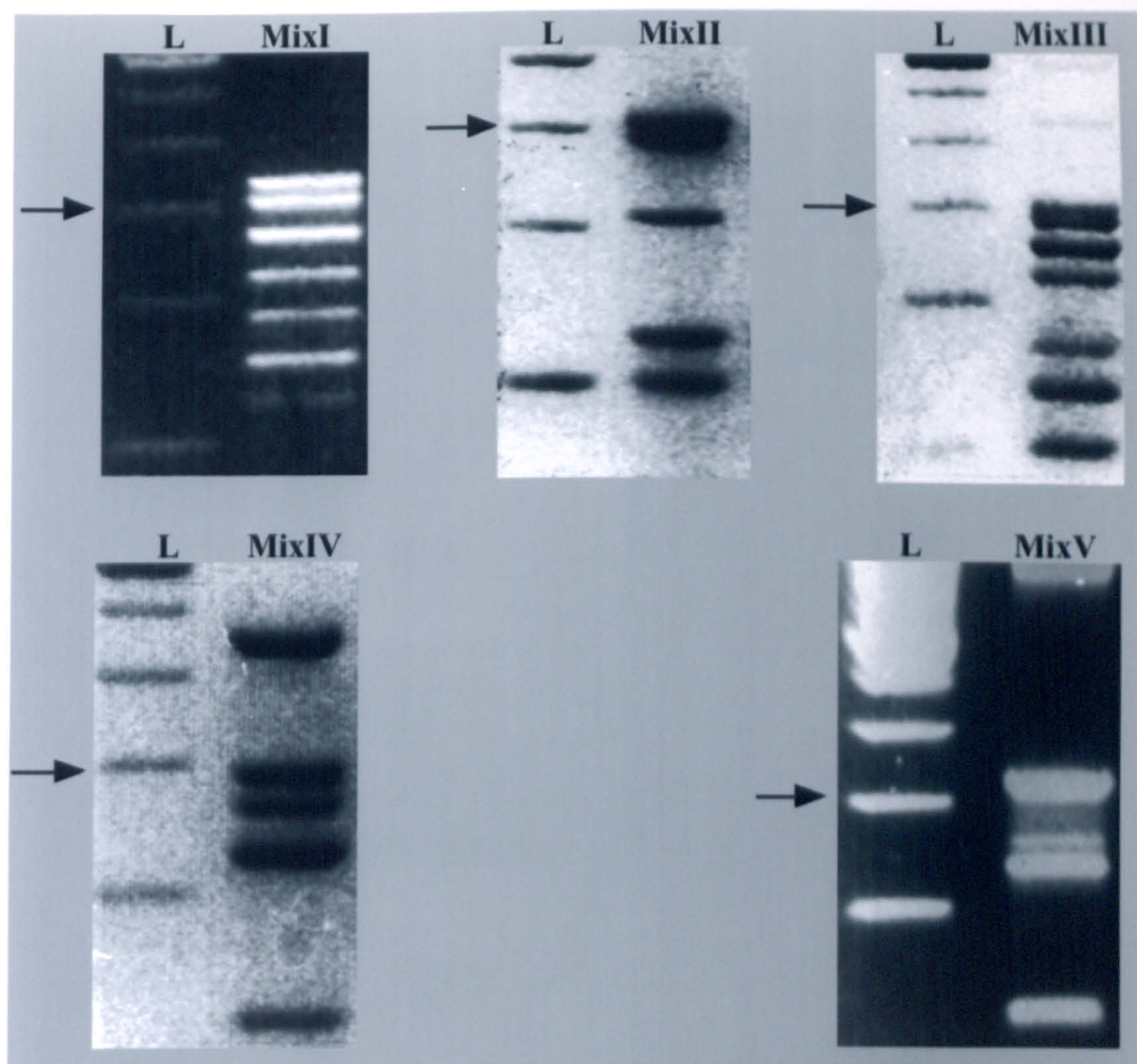


Figure 3.53: Pattern of multiplex PCR analyses of 28 Y-DNA loci. The primers making up each mixture are ordered according to decreasing length of their amplification products as indicated in the table. The PCR products were resolved on an ethidium bromide stained 3% agarose gel (3 parts Nuseive : 1 part Seakem), electrophoresed at 10 volts/cm for 3 hours and visualised under UV light. L = 100 bp DNA ladder. Arrows indicate the 300 bp DNA band.

| MIX I | MIX II | MIX III | MIX IV | MIX V |
|-----------------------|-----------------------|--------------------------|-----------------------|---------------------------|
| 1 = sY84 (326bp) | 1 = sY143 (311bp) | 1 = sY86 (320bp) | 1 = sY14 (472bp) | 1 = pY6BaH34pr (910bp) |
| 2 = sY134 (301 bp) | 2 = sY157 (285bp) | 2 = sY105 (301 bp) | 2 = sY9 (303 bp) | 2 = Fr-IIpr (313 bp) |
| 3 = sY117 (262 bp) | 3 = sY81 (209 bp) | 3 = sY82 (264 bp) | 3 = sY127 (274 bp) | 3 = Y6HP52pr (258 bp) |
| 4 = sY102 (218 bp) | 4 = sY182 (125 bp) | 4 = Y6HP35pr (226 bp) | 4 = sY109 (233 bp) | 4 = Y6HP35pr (226bp) |
| 5 = sY151 (183 bp) | 5 = sY147 (100 bp) | 5 = Y6PHc54pr (166bp) | 5 = sY149 (132 bp) | 5 = Y6D14pr (134 bp) |
| 6 = sY94 (150 bp) | | 6 = sY153 (139 bp) | | |
| 7 = sY88 (123 bp) | | 7 = sY97 (104 bp) | | |

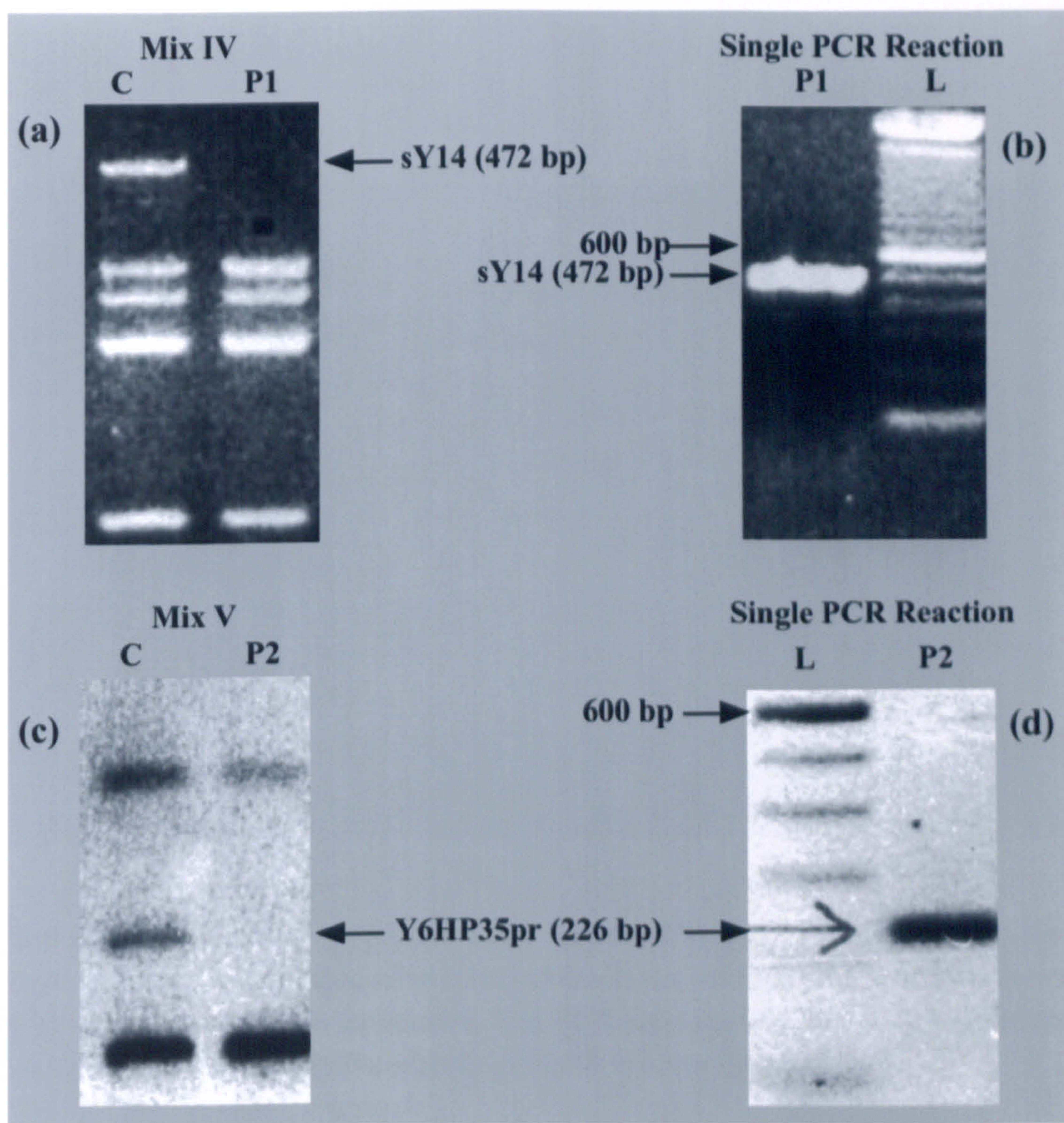


Figure 3.55: Examples of false deletion upon the multiplex PCR screening of 46,XY infertile males. (a) and (c) show the apparently deleted bands in two different patients. Single PCR reaction using the deleted primer(s) showed amplification of the specific segment from the falsely deleted bands in these two patients (b and d). The PCR products were resolved in an ethidium bromide stained 3% agarose gel (3 parts Nuseive : 1 part Seakem), electrophoresed at 10 volts/cm for 3 hours and photographed under UV light. L = 100 bp DNA ladder, C = control, P = patient.

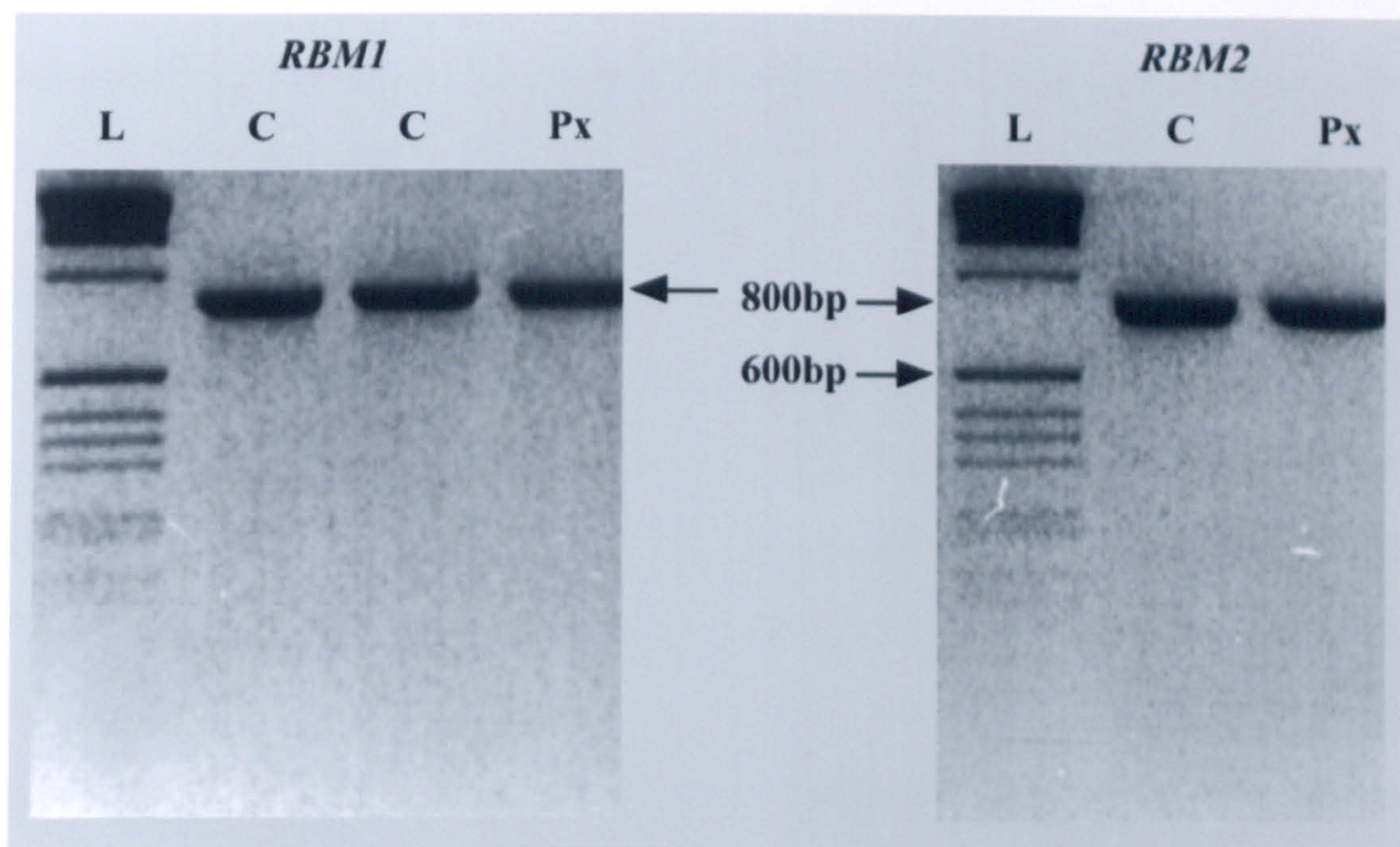


Figure 3.56: PCR analysis of patient's DNA using *RBM1* and *RBM2* gene-specific primers. Patient (Px) who showed microdeletions in the multiplex PCR (Figure 3.54) showed no deletion with these primers. The PCR products were electrophoresed on an ethidium bromide stained 1% agarose gel at 10 volt/cm for 45 minutes. L= 100 bp DNA ladder; C= control

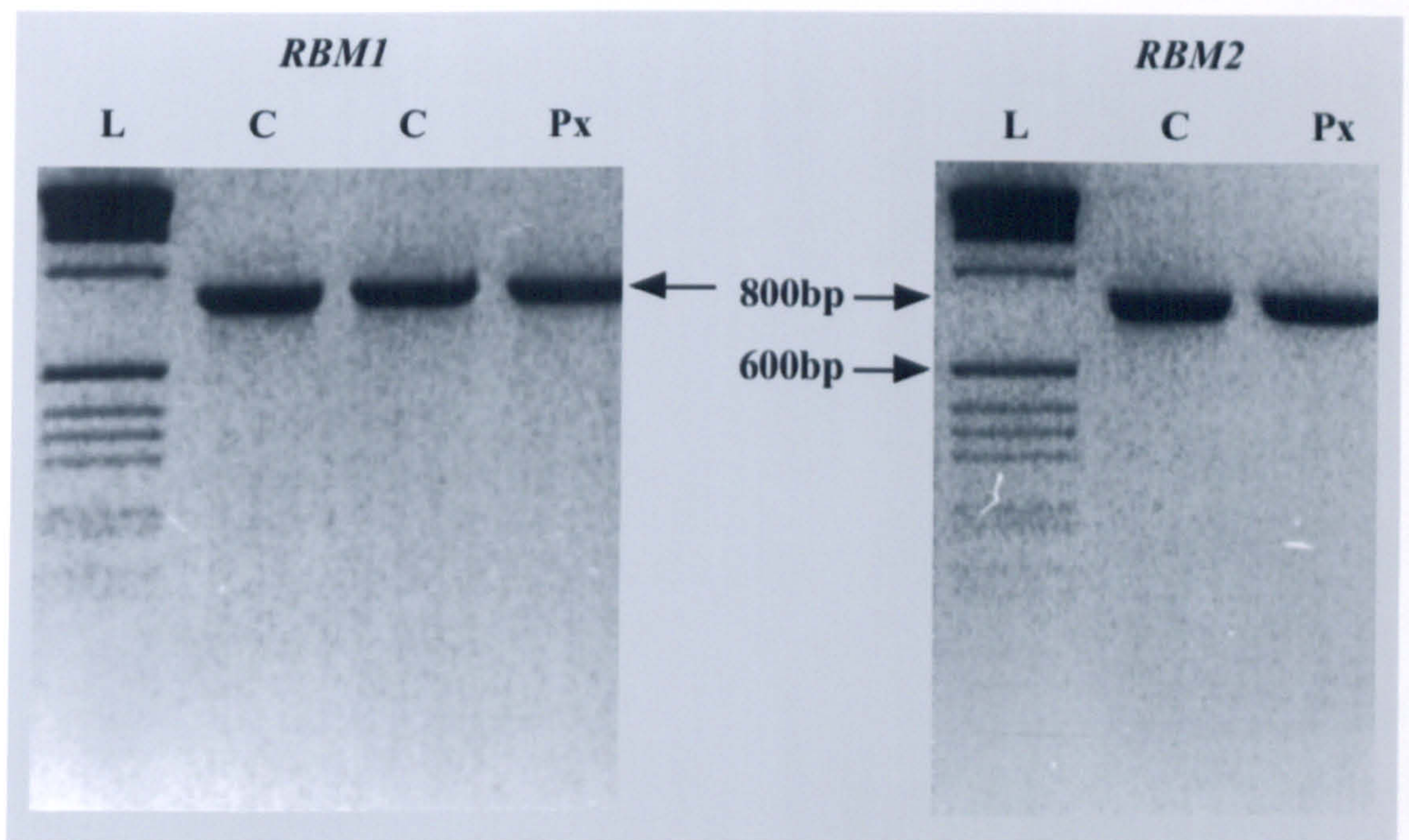


Figure 3.56: PCR analysis of patient's DNA using *RBM1* and *RBM2* gene-specific primers. Patient (Px) who showed microdeletions in the multiplex PCR (Figure 3.54) showed no deletion with these primers. The PCR products were electrophoresed on an ethidium bromide stained 1% agarose gel at 10 volt/cm for 45 minutes.

L= 100 bp DNA ladder; C= control

b) FISH study: - FISH study using probe PHY2.1 of the DYZ2 locus showed normal hybridisation pattern (Figure 3.57) from all screened patients indicating the presence of the Yq12 (interval 7) and confirming the previous cytogenetic findings obtained by Q and C-banding techniques. To detect the presence of deletions in the *RBM1* and *RBM2* genes cDNA probes MK5 and MK29 were used. No such deletion were detected in the studied patients including the patient who showed microdeletion in the Yq11 region by PCR study (Figure 3.57).

3.2.3.3 Mutation analysis of the RBM1 gene

Since the *RBM* genes are a multicopy gene family, Southern blotting analysis was applied to detect gene-copy deletions in all studied 50 idiopathic sterile males. Southern blotting analysis using MK5 probe and two different restriction enzymes (*EcoRI*, *TaqI*) revealed multiple *RBM1* hybridisation fragments from patients' DNA. No abnormal pattern was detected in any DNA sample compared to normal male controls (Figure 3.58). Southern analysis of DNA from the patient who showed microdeletion by PCR could not be performed due to DNA degradation.

To characterise the molecular pathology of the *RBM1* gene in idiopathic sterile males, DNA samples from all studied patients were further investigated for the presence of point mutations in this gene. Exon-intron boundary sequences of the *RBM1* gene A type were kindly provided by Dr. H.J.Cooke, Medical Research Council, Human Genetics unit, Western General Hospital, Edinburgh. He also, generously provided the exon/intron boundaries of exon 2, which is proposed to have an RNA binding motif, in B, C and D copies of the gene. PCR primers to amplify all these exons were designed using the OligTM program and PCR reactions were optimised to get single specific amplification product from each of them (Figure

3.59a and b). Screening for point mutation in the *RBM-1* gene A type exons 2-11 and in exon 2 of B, C and D gene copies was performed by means of the SSCP analysis. Radiolabelled PCR amplifications of the specific exons were analysed by non-denaturing 0.5X MDE gel electrophoresis with and without 5% glycerol at room temperature. Few false positive results were detected which disappeared upon second gel electrophoresis of the same samples (Figure 3.60). No other positive SSCP results were obtained.

For exon 12 (437 bp) of the *RBM* gene heteroduplex analysis using 1X MDE gel was performed to screen for point mutation in that exon from all screened patients. No abnormal band pattern was detected in comparison to normal control (Figure 3.61).

3.2.3.4 Microdeletion screening of the DAZ (Deleted in Azoospermia) gene in idiopathic sterile males

Recently, Reijo et al (1995) reported a new gene called *DAZ* that was deleted in some azoospermic idiopathic sterile males. They also, refined the physical map of the AZF region deleted in the azoospermic men. Using this information, the 50 azoospermic and severe oligozoospermic infertile men included in this study were further screened for microdeletion in Yq11 using 19 single STS PCR primer sets, included two sets (sY254, sY255) within the *DAZ* gene and one *DAZ* gene-specific PCR primer set. No further patients with deletions were detected in the screened cohort. The patient who showed microdeletion upon multiplex PCR analysis showed deletion of the *DAZ* gene-specific PCR primer set and of 16 STS PCR primer sets including the two sets within the *DAZ* gene (Figure 3.63). The deletions in this patient were found to span the area between DYS232 and DYS239 which is localised to intervals 6C and 6F on the distal part of Yq11 (Figure 3.64).

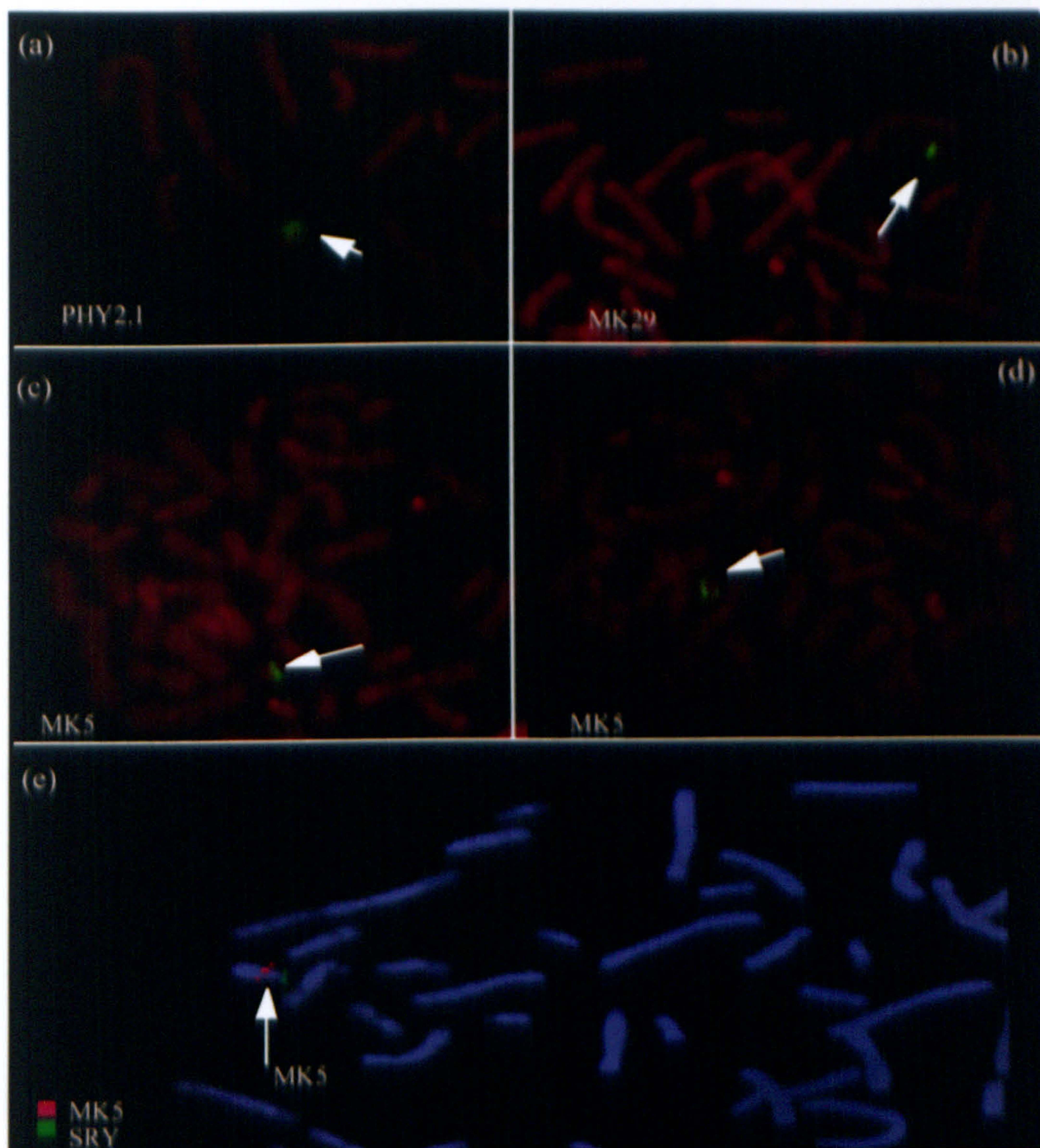


Figure 3.57: (a) Example of FISH study using PHY2.1 probe hybridised to metaphase spread from infertile male. Clear signals detected at the heterochromatic region of the Y chromosome. (b-d) FISH analysis of MK5 and MK29 cDNA probes to metaphase spreads of infertile males. Signals of hybridisation (arrows) are seen exclusively on the Y chromosome at the interface between heterochromatin and euchromatin. (e) Partial metaphase spread from the patient who showed microdeletions by PCR. Normal hybridisation of the MK5 probe was detected (arrow). The green signals in (e) represent hybridisation of the SRY probe to the Yp region. The chromosomes were counter stained with PI (a-d) and with DAPI (e).

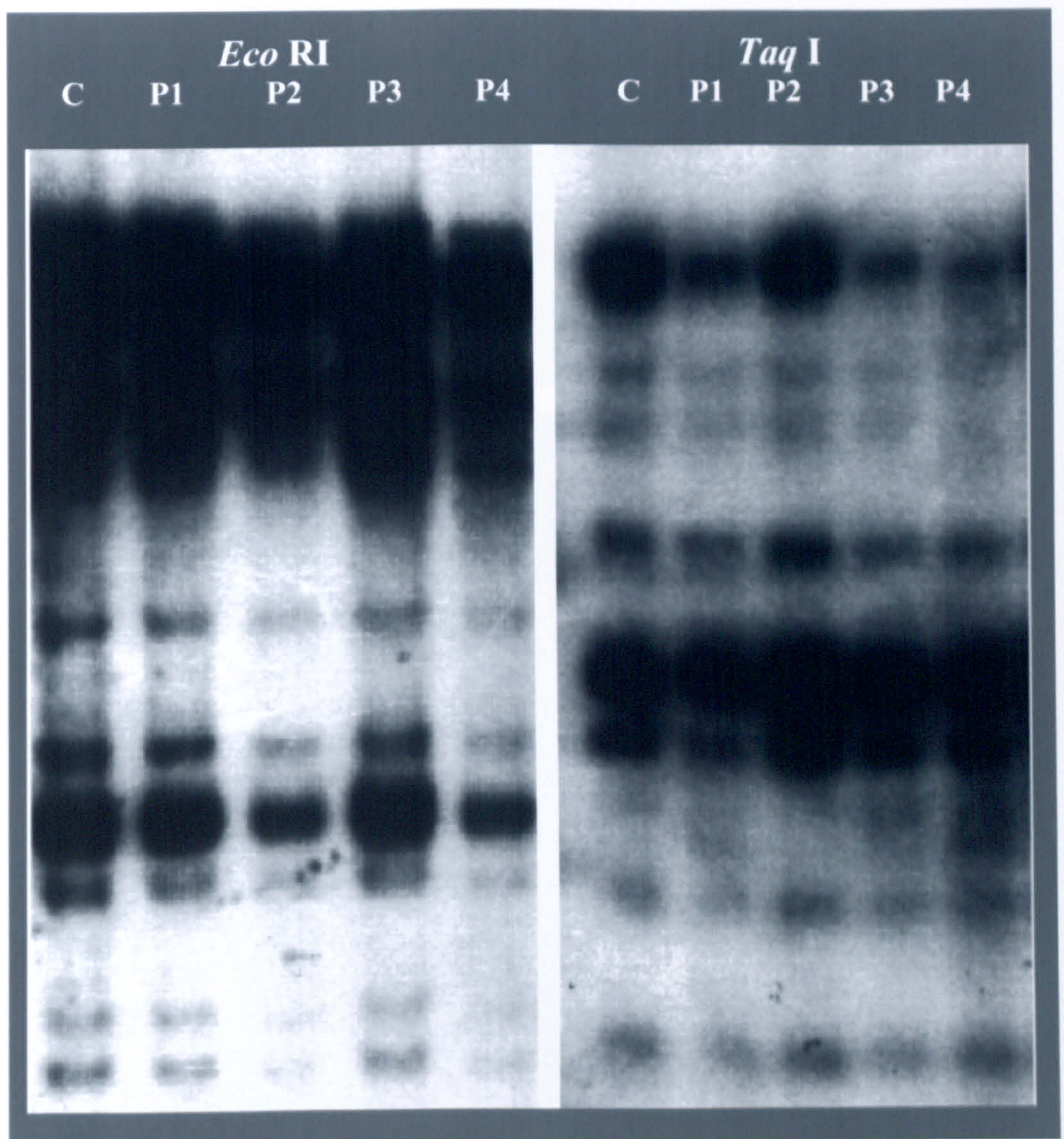


Figure 3.58: Examples of Southern blot analysis using Mk5 cDNA and two different restriction enzymes (*Eco*RI and *Taq*I). Male genomic DNA hybridised with the *RBM1* (Mk5) sequence demonstrated multiple locations of the *RBM* gene. No deletion(s) was detected in any screened patient. The digested fragments were separated in 0.8% agarose gel containing 0.5 µg/ml ethidium bromide and electrophoresed at 2 volts/cm for 16-20 hours.
P = patient, C = control.

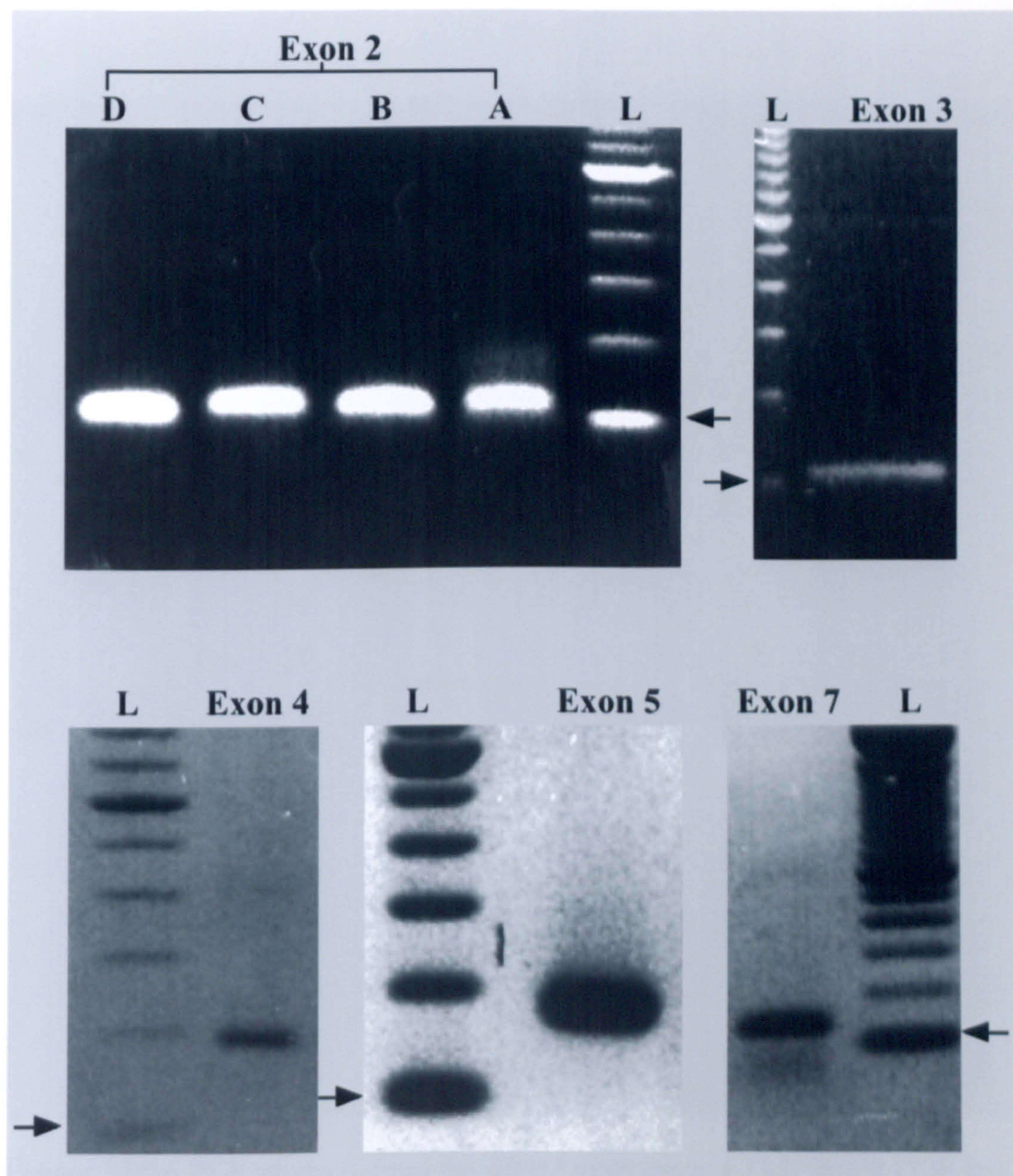


Figure 3.59a: PCR products from exon 2 of the *RBM* gene family (A, B & C = 113 bp, D = 100 bp) and from the *RBM1* gene exons 3 (128 bp), 4 (190 bp), 5 (178 bp) and 7 (125 bp). The PCR products were run in an ethidium bromide stained 1% agarose gel and electrophoresed at 10 volts/cm for 45 minutes. Arrows indicate 100 bp band. L = 100 bp DNA ladder.

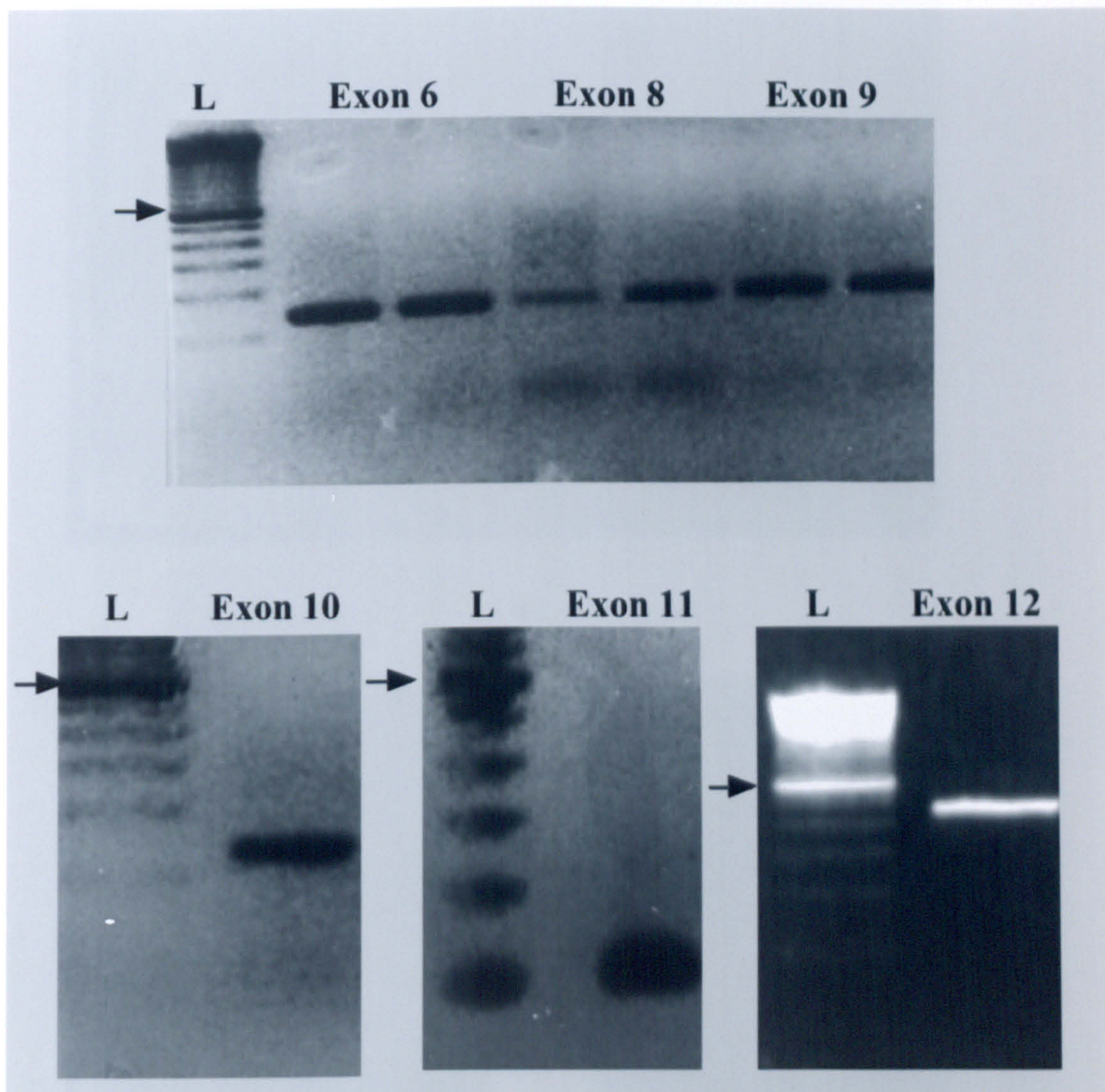
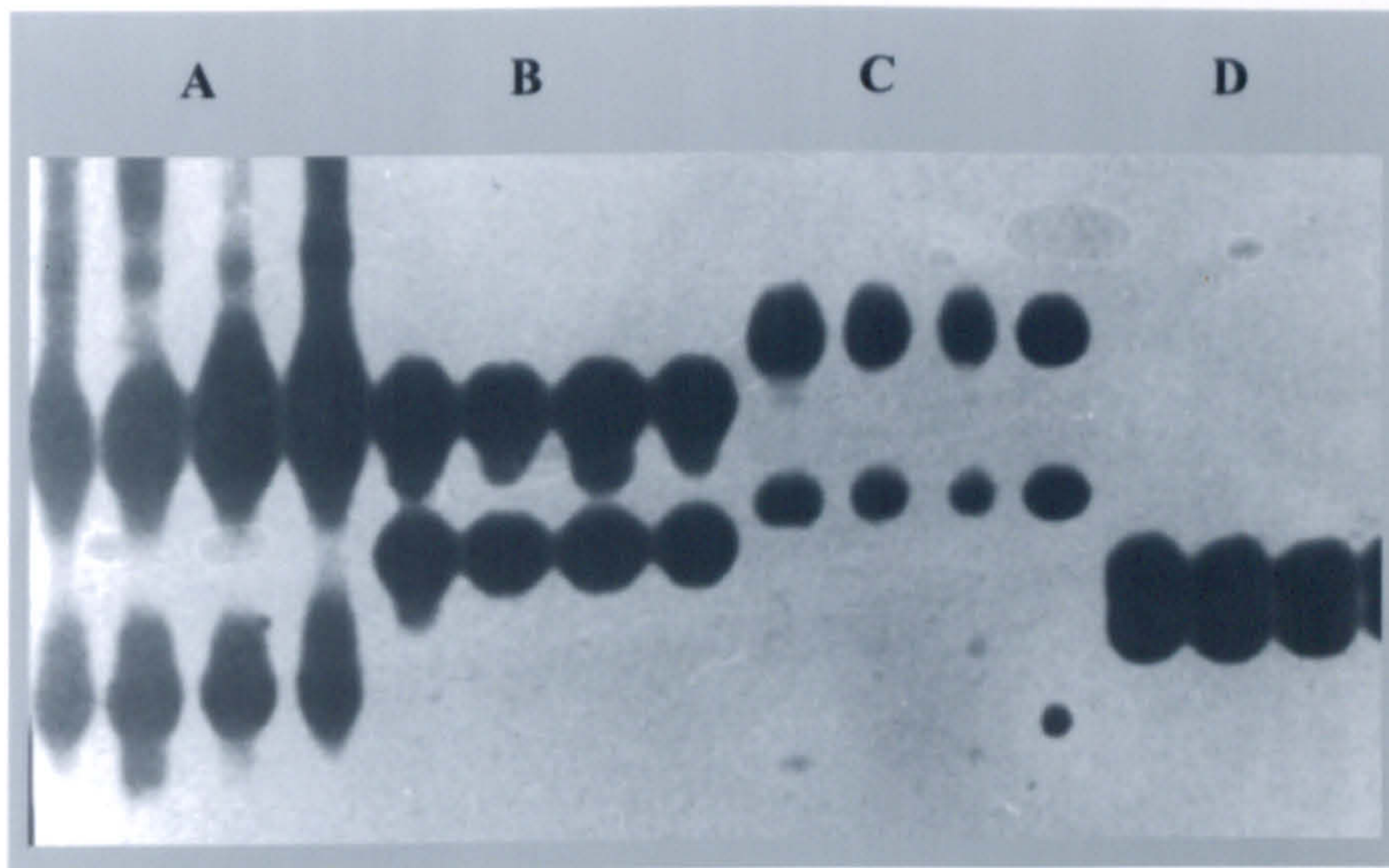


Figure 3.59b: PCR products from the *RBM1* gene exons 6 (119 bp), 8 (136 bp), 9 (150 bp), 10 (127 bp), 11 (105 bp) and 12 (437 bp). The PCR products were run in an ethidium bromide stained 1% agarose gels at 100 volts for 45 minutes, visualised and photographed under UV light. Arrows indicate 600 bp band. L = 100 bp DNA ladder.

(a)



(b)

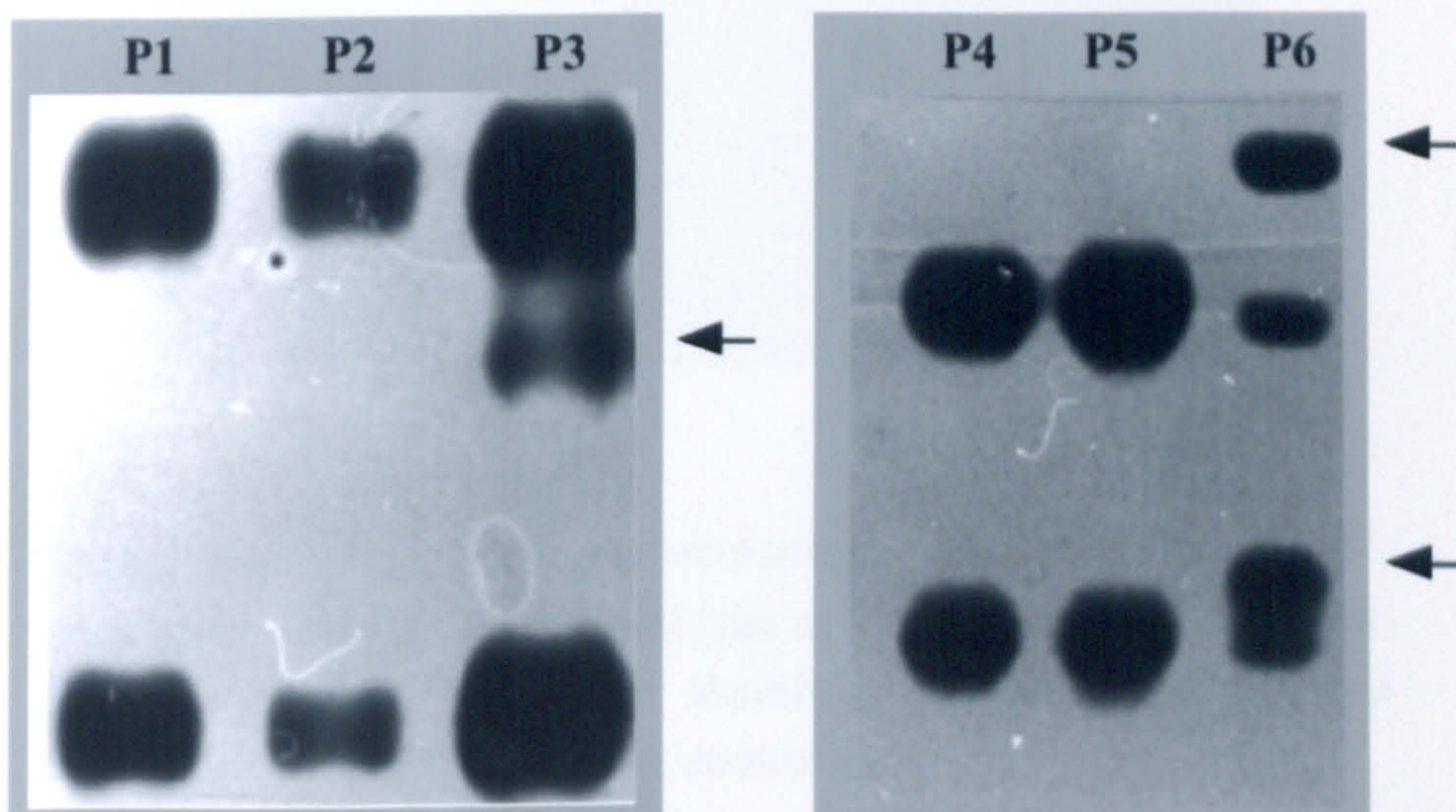


Figure 3.60: (a) Examples of radiolabelled SSCP analysis of the *RBM1* gene exon 2 (A, B, C and D) copies. (b) False positive (arrows) results of SSCP analysis of exon 3 obtained in two different patients. The PCR products were run in 0.5X MDE gel containing 5% glycerol and electrophoresed in 0.6X TBE buffer at 5 Watts for 16 hours at room temperature.

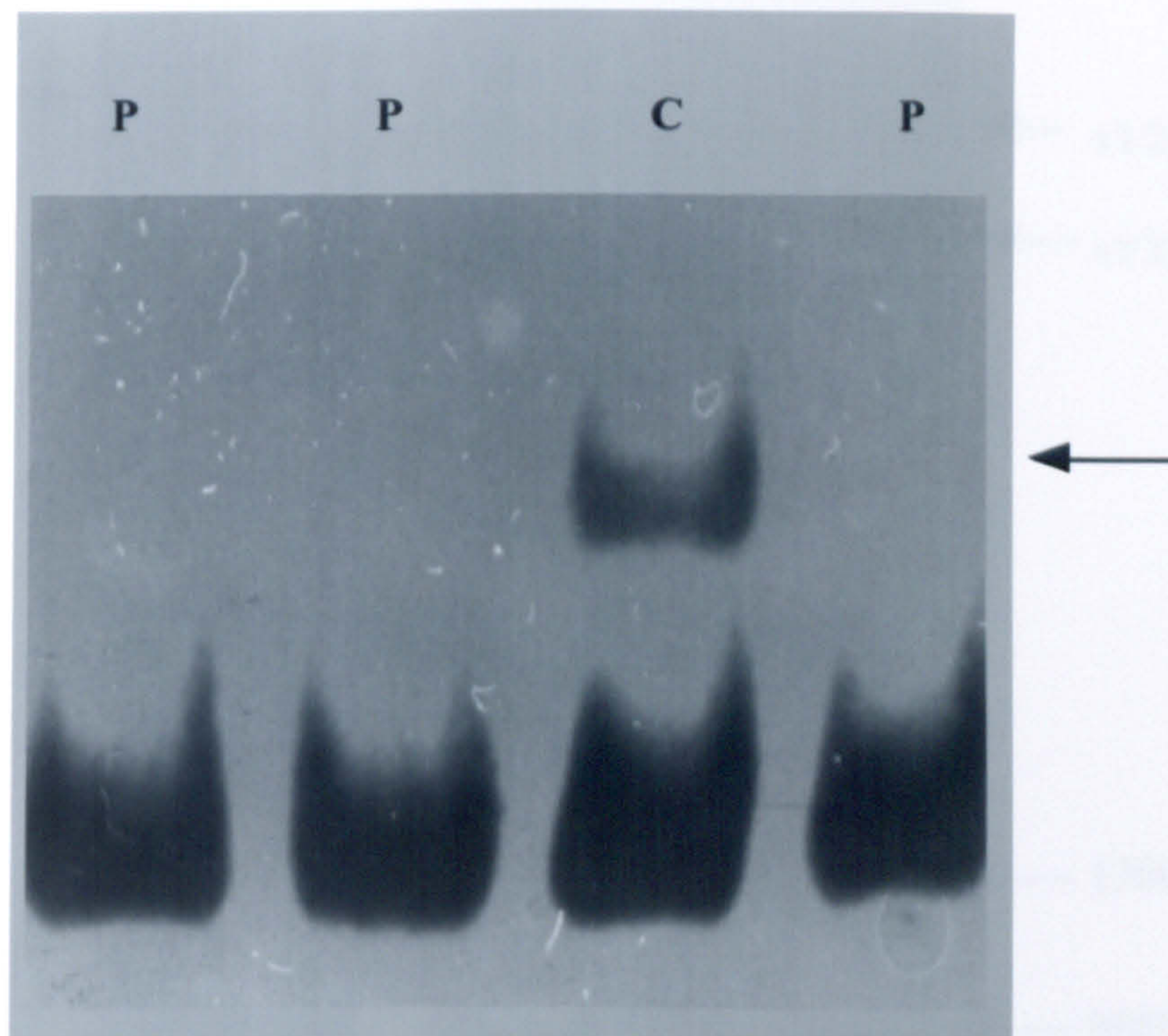


Figure 3.61: Example of the heteroduplex analysis of the *RBM1* gene exon 12 on a 1X MDE gel (run at a constant 800 volts for 18 hours) detected by silver staining. Arrow indicates the heteroduplex band of a positive control sample.

P = patient; C = positive control.

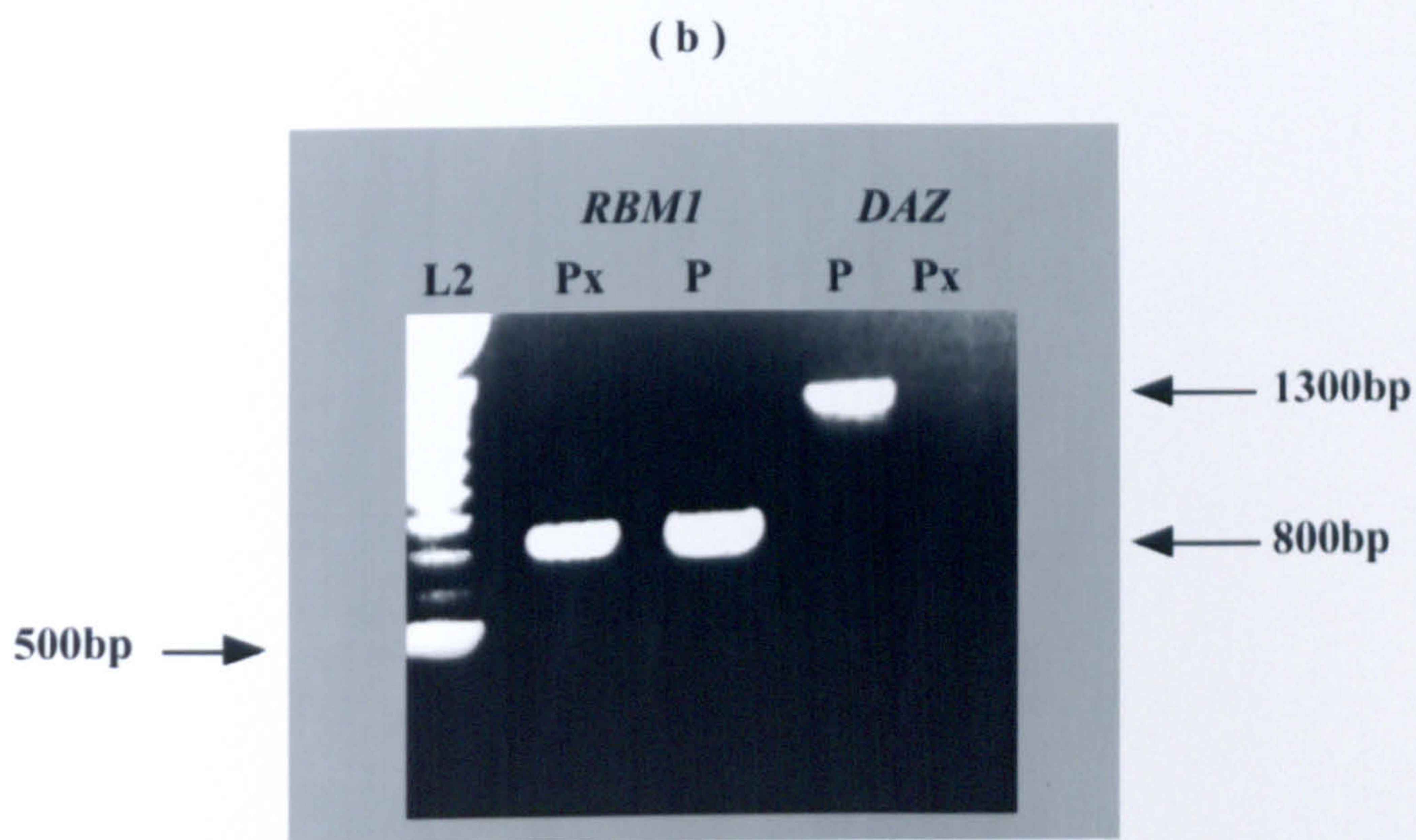
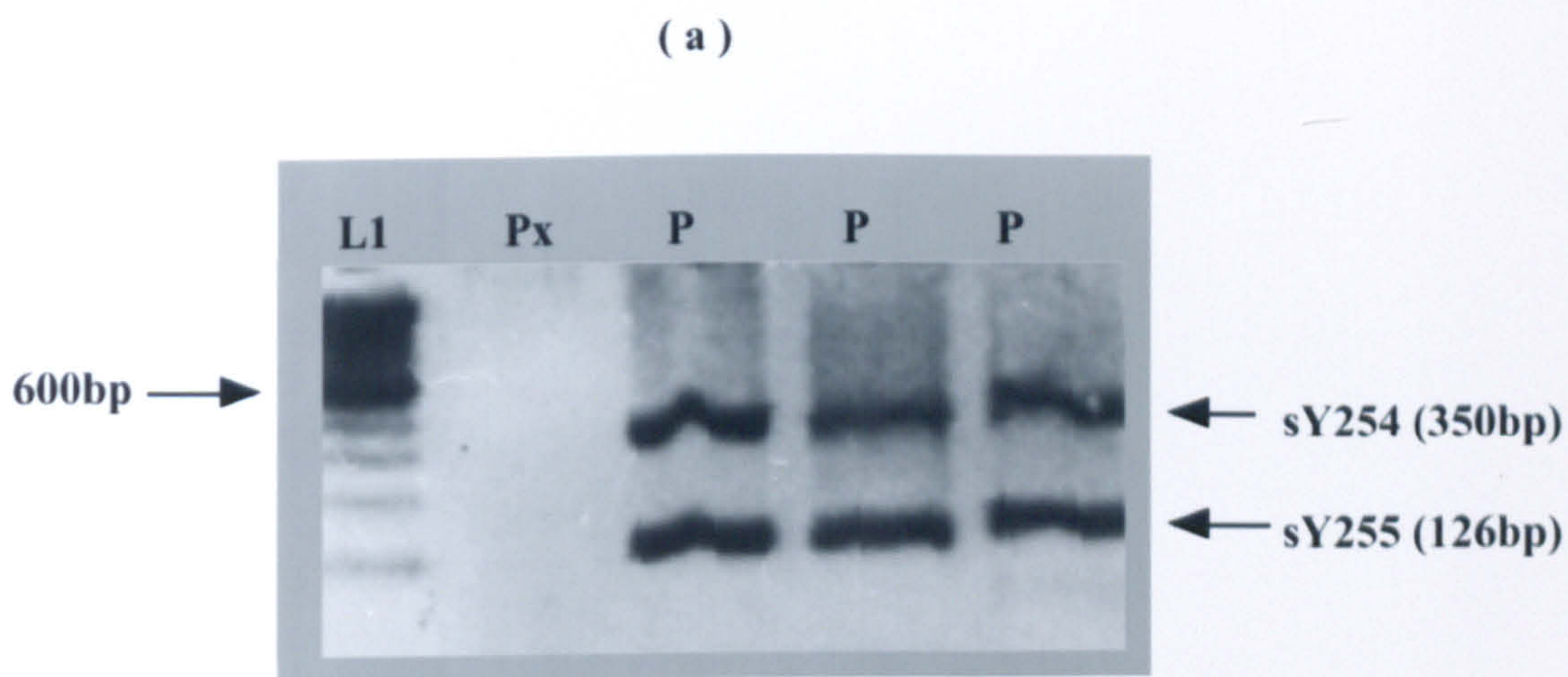


Figure 3.62: (a) Multiplex PCR analysis of DNA from infertile men using two primer sets within the *DAZ* gene (sY254 and sY255). (a) only the previous patient (Px; Figure 3.54) showed deletion of both primer sets. (b) PCR analysis of the patient (Px) showing deletion of the whole *DAZ* gene but no deletion of the *RBM1* gene. The PCR products were resolved on an ethidium bromide stained 1% agarose minigel for 45 minutes at 10 volt/cm. Px= patient with microdeletion; P= patient; L1= 100 bp DNA ladder; L2= 1 Kb DNA ladder.

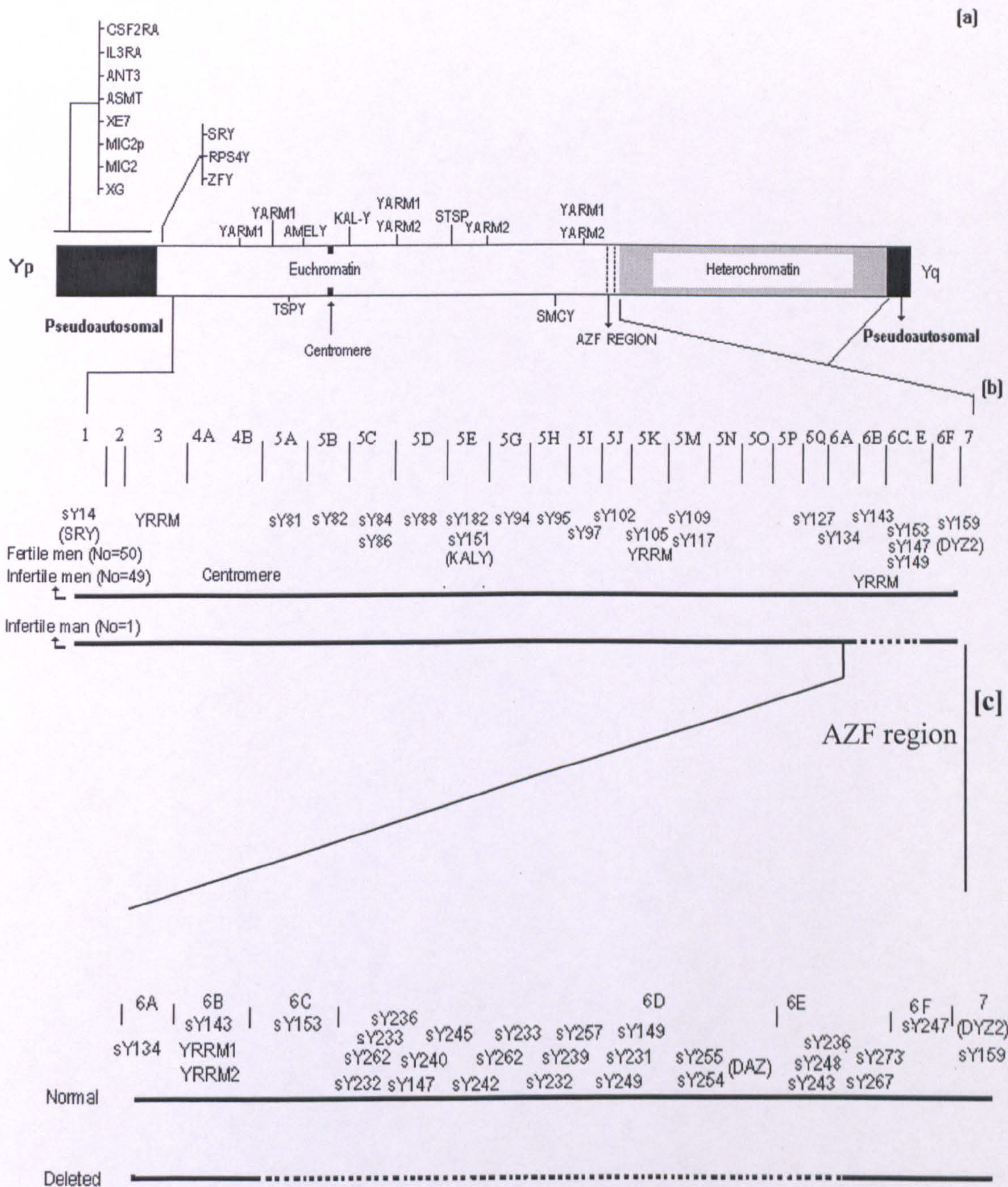


Figure 3.63: Deletion map of the AZF region on human Y-chromosome. (a) Diagram of the human Y chromosome. (b) Low resolution analysis of Y chromosome of one case of azoospermia with microdeletion. Along top border are listed deletion intervals 1-7 and, immediately below, 22 STS and YRRM loci. Shown below are results of tested fertile and infertile men for the presence (solid black line or absence (minus sign) of each STS. (c) Higher resolution map of AZF region. Along top border are listed the STS intervals used in this study. Below are shown the results of the deletions detected on the same patient. (Reijo et al 1995 with modifications).

CHAPTER 4

DISCUSSION

(4) DISCUSSION

Detection of mutations is one of the most important areas of molecular biology and it plays an important role in the diagnosis of human inherited diseases (Cotton R, 1993; Grompe M, 1993). The present study focused on application of different screening strategies that could detect large or minor sequence alterations in patients with sex chromosomes aberrations, sex determination (e.g. in the *SRY* gene), sex differentiation (e.g. *AR* gene) and in male patients with non obstructive infertility (*RBMI* gene). These strategies involved the application of cytogenetic, molecular cytogenetic (FISH) and molecular investigations. The spectrum of the investigated mutations ranged from cytogenetically visible chromosome abnormalities to microdeletion and finally to single base alteration (Table 4.1).

4.1 Cytogenetic and molecular cytogenetic studies

In this study the 24 cases included had different clinical manifestations and chromosomal abnormalities identified by standard cytogenetic techniques (Giemsa banding, G-banding, late replication of X). Two thirds of these patients (16/24) were ascertained because they had phenotypes suggestive of Turner syndrome or its variants. Reassessment of the chromosomal constitution of these patients using fluorescent in situ hybridization technique was carried out to delineate and characterize the chromosomal abnormalities more precisely.

In only 4/24 (16.7%) cases were the cytogenetic findings fully consistent with the FISH results (one case of 46,XX, one with 48,XXXX, and two cases with 45,X/46,XY karyotype). In the remaining 20 cases (83.3%) application of FISH yielded additional information not available by the standard cytogenetic method.

Mosaicism of X and Y chromosomes and structural rearrangements of the sex chromosomes are two of the major causes for gonadal dysfunction, infertility or abnormal sexual development in both males and females (Therman and Susman, 1990; Hsu L, 1994; Ogata and Matsuo, 1995).

Table 4.1: Type of mutations detected in the present study and other studies.

| Disorders | No | Investigations | Results in the present study | Other studies | References |
|---|-----|-----------------------------------|---|---|--|
| Known or suspected Sex chromosome abnormalities | 24* | Cytogenetics FISH | 4 cases (16.7%) had consistent cytogenetic and FISH findings. In 20 cases (83.3%) FISH gave additional information not available by cytogenetic methods such as: Determination of the origin of the abnormal chromosome, Detection of cryptic rearrangements, Detection of cryptic mosaicism, Determination of the breakpoints of the abnormal chromosome | The additional findings of FISH technique have been reported by other authors | Hsu L, 1994 Ogata and Matsuo, 1995 Fernandez et al, 1996 Jacobs et al, 1997 Dalton et al, 1998 Fernandez et al, 1996 Turner et al, 1998 Zhang et al, 1997 |
| Sex-disorders | 13 | | | | |
| (a) 46,XX males | 4 | Cytogenetics FISH Molecular | Three cases were <i>SRY</i> positive (75%) detected by FISH and PCR studies. FISH study revealed the transferral of the Y material to the short arm of the X chromosome in 3 cases | 80% of XX males are <i>SRY</i> positive Most of the reported cases showed t(Xp;Yp) by Southern blot and isotopic in situ hybridisations, however few reports used FISH technique | Affaraer al, 1987; McElreavey et al 1993a Weil et al, 1994 Van der Auwera et al, 1992 Vilain et al, 1994 Ester et al, 1998 |
| | | | One case was <i>SRY</i> negative (25%) | 10%-20% of XX males were reported to be <i>SRY</i> negative | McElreavey et al 1992a McElreavey et al 1993a Lopez et al, 1995 |

| | | | | | |
|--|----|-----------------------------------|--|---|---|
| (b) 46,XY females Androgen Insensitivity Syndrome | 5 | Cytogenetics Molecular | A G 4109→T transversion causes Val 889 Leu in exon 8 of the <i>AR</i> gene detected by SSCP analysis in one patient with CAIS. | The same given codon replacement of Val 889 by other amino acid in the <i>AR</i> gene were reported | Pinsky et al, 1992 De Bellis et al, 1994 Gottlieb et al, 1996. |
| (c) XY gonadal dysgenesis | 4 | Cytogenetics FISH Molecular | No mutation detected in the <i>SRX</i> gene. This could be due to small sample size | Only 10-15% of the XY females have mutation in the <i>SRX</i> gene and to date, 24 mutations have been reported | Schmitt-Ney et al, 1995 Kwok et al, 1996 Veitia et al, 1997 |
| Male infertility | 56 | Cytogenetic | 5/56 cases (8.9%) had abnormal karyotype. Four of them (7.1%) had 47,XXY and one (1.8%) had 47,XXY karyotype | The incidence of abnormal karyotypes reported among infertile men varies from 2.2%-19.6%. The 47,XXY karyotype is the most frequent | Chandley A, 1979 Pandiyar et al, 1996 Gundez et al, 1998 McLaren A, 1998 |
| Idiopathic 46,XY Infertile men (for 50 out 56 cases) | 50 | FISH Molecular | No microdeletion of the <i>RBM1</i> gene using FISH and Southern blot hybridisation were detected in any screened patient | Deletion of the <i>RBM1</i> gene in infertile men were reported | Ma et al, 1993 Kaboyashi et al, 1994 |

| | | | | |
|-------|----|---|--|---|
| | | <p>Multiplex PCR</p> <p>1/50 (2%) of the idiopathic 46,XY infertile male had a microdeletions in Yq11 region between DYS232 and DYS239 (interval 6C-6F) detected by multiplex PCR</p> | <p>Microdeletions reported in 3-18% of idiopathic infertile males</p> | <p>Ma et al, 1993 Vogt et al, 1996 Meschede and Horst, 1997</p> |
| | | <p>The patient retained the <i>RBM1</i> gene but had deletion of the <i>DAZ</i> gene</p> | <p>Deletions in the <i>DAZ</i> gene were reported frequently</p> | <p>Reigo et al, 1995 Stupia et al, 1996b Vogt et al, 1995; 1996</p> |
| | | <p><i>RBM1</i> gene screening</p> <p>No point mutation could be detected using SSCP and HA analyses of the <i>RBM1</i> gene</p> | <p>Polymorphism in exon 2 was detected in one patient in one study</p> | <p>Pressor et al, 1996</p> |
| Total | 93 | | | |

* Refer to Table 3.1 for detailed description of chromosomal abnormalities

Since the first cytogenetic report of 45,X karyotype in females with Turner syndrome (TS) by Ford et al (1959), a variety of numerical and structural abnormalities of X and Y chromosomes have been reported. Recent studies have confirmed that the majority of cases with 45,X demonstrates mosaicism as confirmed by applying molecular techniques. These individuals present with varying degrees of clinical and pathological manifestations (Therman et al, 1990; Ogata and Matsuo, 1995). At conception, loss of an X chromosome resulting from meiotic nondisjunction is lethal. Almost all females with 45,X karyotype have gonadal dysgenesis, with few exceptional cases, in whom the possibility of mosaicism (45,X/46,XX) cannot be absolutely excluded (Zinn et al, 1993; Jacobs et al, 1997). Since the development of banding techniques there is a consensus that a minimum of 20 cells or even more should be scored to exclude low grade mosaicism. Recently, application of FISH (X specific probes) has confirmed that ~99% of the Turner syndrome cases are mosaic and the pure 45,X cell line is very rare (Fernandez et al, 1996; Jacobs et al, 1997). The high percentage of fetal and embryonic miscarriage with 45,X karyotype points to the necessity of mosaicism for survival (Hook and Warburton, 1983). Routine analysis of 20-30 metaphases has limited the ability to exclude low grade mosaicism. The rate of detection of mosaicism is affected by many factors; (a) tissue types and number of tissues analysed, number of cells analysed, (b) sensitivity of the techniques applied, (c) cell selection, leading to disappearance of cell lines (Fernandez et al, 1996). The most frequently observed mosaicism is 45,X/46,XX, occurring in about 30-35% of the cases (Fernandez et al, 1996). Mosaicism for structural abnormality of one of the X chromosomes (isochromosome for Xq or Xp) has been reported to occur in 20-25% of cases and an unidentified 'marker' chromosome or fragment (der X or Y) has been observed in 12-15% of the cases (Fernandez et al, 1996). Application of molecular techniques such as FISH and PCR substantially improves the rate of detection of low frequency cell lines and possible structural rearrangements.

The first advantage of application of FISH technique is to confirm the cytogenetic finding especially in an unusual situation. For instance, the female in case # 16 with short stature and solitary right kidney was thought to have a 46,XX karyotype by routine cytogenetics. Application of FISH using whole X and Y

chromosomes paint and centromeric probes for X and Y chromosomes did not show any mosaicism or structural rearrangement of the X. FISH study excluded the presence of Y material in this case. Thus, application of FISH technique has been useful in excluding low grade mosaicism in this patient who presented with short stature and some stigmata of Turner phenotype.

A young female (case # 24) with minor dysmorphic features and multiple congenital anomalies was confirmed to have 48,XXXX karyotype. Whole chromosome painting of X and centromeric probe for X analysis in this patient confirmed 4 copies of the X chromosome. No mosaicism was detected. Late replication studies using BrdU technique showed 3 inactive X chromosomes. This case was investigated as a positive control for X inactivation analysis as well as to ascertain the specificity of the X chromosome specific probes. Females with tetra X resulting from post zygotic nondisjunction have been reported with clinical features of microcephaly and dysmorphic features (Nielsen et al, 1977; Therman and Susman, 1993).

Marker or ring X{r(X)} chromosomes of varying size have been reported in 5% of Turner syndrome patients (Jacobs et al, 1997). The phenotypic effects of the r(X) chromosomes are variable. Some had typical Turner syndrome phenotype, while others had a more severe phenotype including mental retardation, and other abnormalities (Cole et al, 1994). The more severe phenotype was usually associated with very small r(X) chromosomes which did not include the X-inactivation (XIST) locus (Migeon et al, 1993; Janie et al, 1995). Presumably, the abnormal phenotype was due to functional disomy for the duplicated X chromosome regions (Callen et al 1995; Tumer et al, 1998). However, not all the findings were in favor of this suggestion. Severe phenotypes were also observed in patients with late replicating markers (Dennis et al 1993). Regardless of the inclusion of XIST locus on the mar (X), clinical findings of the 45,X/46,X,+mar(X) karyotype differ from those found in the present study (Tumer et al, 1998).

The second advantage of FISH application is to identify the nature of the marker or unknown chromosome. This goal was achieved in the first nine cases (cases #1-9) included in the present study. In all these cases the nature of the ring chromosome was identified and proved to be X chromosome derived with no Y material. In addition, application of the FISH technique demonstrated the presence

of the XIST locus in the 7 patients tested (cases # 1-7) with ring X chromosome. In Turner patients with small ring X (cases # 1-3) in the present study, DXZ1 (centromeric) and XIST (Xq13) were present while DMD (Xp21) and KAL (Xp22) were absent, suggesting the breakpoints in these 3 patients to be proximal to Xp21 and distal to Xq13. Due to lack of other pericentromeric probes suitable for FISH analysis, finer assessment of the breakpoints were not possible. Tumer et al, (1998) by applying FISH have reported two patients with small ring (Xs) with breakpoints Xp11.23-Xq11.2 and Xp11.23-Xq11.1. In the same respect, Callen et al, (1995) reported two patients with small ring (Xs) with the breakpoints of Xp11.21-Xq12 and Xp11.21-Xq13. Recently, a breakage hot spot in a 3 Mb region of Xp11.21 has been suggested (Wolff et al, 1994). Based on the presence of this hot spot at Xp11.21 and on the comparison of the ring size in the present cases and of those reported ones, the short arm breakpoint of these 3 cases with small rings is most likely to be at Xp11.21. However, further studies defining the Xp breakpoints of these 3 cases are needed.

Four cases of the present study had medium size ring (Xs). Three of them (cases # 4, 6 and 7) showed the presence of KAL (Xp22) and XIST (Xq13) loci and absence of DXZ4 (Xq24) so demonstrating the breakpoints to be distal to Xp22 and proximal to Xq24. The last case (# 5) gave positive signals with DXZ1, XIST and DXZ4 and negative signals with the short arm probes (DMD and KAL) suggesting the site of the breakpoints to be proximal to Xp21 and distal to Xq24.

The XIST gene is a candidate for a gene which determines X inactivation since it is only expressed from the inactive X, and maps to the region known to contain the X inactivation region (Brown et al, 1992). Ring chromosomes containing XIST are likely to contribute to the patient's phenotype. When XIST is absent, the euchromatin of the marker will be active resulting in a disomic expression of normal genes. The subsequent over expression and interactions of these genes is the genetic basis for the abnormal phenotype.

It is now established that patients with a small ring marker derived from the X chromosome in association with a 45,X karyotype show the typical Turner phenotype if the X inactivation centre is present (Callen et al, 1995). In the present work late replication studies of 7 cases with r(X) (cases # 1-7) showed that some of the r(X) were early replicating, in the range of 12%-54%, while others were late replicating ranged from 46%-88%. In all seven cases the normal

X in the cell line containing the r(X) was always active. However, no phenotype/genotype correlation could be made for those cases with r(X) and the size of the ring or the replication pattern. This is due to the presence of the 45,X cell line, which invalidates the attempts of genotype-phenotype correlation (Ogata and Tsutomu, 1995). On the other hand the lack of additional phenotypic abnormalities might be attributed to inactivation of the ring (X) (Callen et al, 1995). Although an association of ring (X) with mental retardation has been previously reported in Turner syndrome (Cole et al, 1994), the nine cases with ring (X) in the present study were associated with a typical Turners phenotype with normal mentality.

The third advantage gained from FISH application is the precise identification of the breakpoint of chromosomal rearrangement. This was obvious in the cases #10 and #11 where the duplicated region has been verified precisely. These two cases represent a very rare cytogenetic finding with 46,X, dir dup(X) (q11.2→q22.3) and mos 45,X/46, inv dup(X) (q28→q22.2) respectively. Reassessment of their karyotype by applying FISH technique using whole chromosome paint (WCPX) and X-specific probes, XIST (Xq13), DXZ1 (centromere), DMD (Xp21) and DXZ4 (Xq24) has precisely verified the duplicated region in each case. In case #10 two copies of XIST and one copy of DXZ4 loci on the long arm of the abnormal X chromosome were observed indicating duplication of the segment q11.2→q22.3. In case #11 FISH analysis confirmed the presence of 45,X cell line and duplication of the segment Xq22.1→q28. However, a more precise assignment of break points could have been possible by using single copy probes for the segment Xq25-Xq28 or telomeric specific probes. Direct or inverted duplication of the long arm of the X chromosome is very uncommon and has been reported in only 25 cases (Garcia-Heras et al, 1997; Zhang et al, 1997). In the majority of cases the rearrangement is maternally inherited (Zhang et al, 1997). In only two cases was mosaicism reported, suggesting a post-zygotic error in the carrier mother. Alternatively an inverted duplication may have resulted from an unequal interchange (crossing-over) between homologous chromosomes (Zhang et al, 1997). Dup(X) in males have been often associated with severe growth and mental retardation, hypoplastic genitalia (undescended testes) and hypotonia (Yokoyama et al, 1992). In females,

dup(Xq) is associated with gonadal dysgenesis, short stature, minor anomalies and developmental delay. These phenotypic features are probably due to expression of genes on the active X chromosome, imprinting effect and/or gene dosage effect (Aughton et al, 1993; Zhang et al 1997). In the majority of non mosaic cases with dup(Xq), a pre-zygotic origin is suggested by the absence of a normal cell line. Aughton et al (1993) have suggested that the clinical effects of dup Xq reflect the extent of duplication and the break points. Larger Xq duplications have been exclusively reported in females (Zhang et al, 1997). Females with dup(X) may present with apparently normal phenotype and or minor anomalies/delayed mental development (Ogata and Matsuo, 1995). The pathogenesis could be attributed to the replication pattern in the different tissues and/or mosaicism. De novo inversion duplications are an extremely rare occurrence. Sporadic cases with dup Xq13.1 →Xq21.2 seem to have no effect on the fertility in females (Garcia et al, 1997; Zhang et al, 1997). Parental origin of the der(X) could also contribute the phenotype in de novo duplications of Xq.

Application of FISH in case # 15, presenting with the stigmata of Turner syndrome and 46,XXp-(pter-p11) karyotype, identified the nature of the derivative chromosome, excluded the presence of mosaicism and detected the breakpoints. FISH study has shown that this patient has deletion of the whole short arm of the X chromosome. Patients with terminal Xp deletions have short stature and may have some somatic traits of Turner syndrome while gonadal function is generally preserved (James et al, 1998). Short stature has recently been shown to be a result of the deletion of a homeobox gene SHOX, which escapes X-inactivation and is located in the pseudoautosomal region Xp (Rao et al, 1997). Regions of both Xp and Xq are thought to carry genes involved in normal ovarian function. Recently it has been proposed that the gene DFRX located at Xp11.4 may play such a role (James et al, 1998). The genes responsible for the other somatic features of the Turner phenotype have not been identified. Studies of patients with deletions of Xp have led to the suggestions that a gene (s) responsible for the stigmata of Turner syndrome may lie proximal to Xp11.2 or along the length of Xp and in Xq21-q26 (Ferguson-Smith, 1991; Rao et al, 1997).

Isochromosome of the short arm of the X chromosome [i(Xp)] does not seem to be viable (Dalton et al, 1998). Indeed monocentric constitutional i(Xp)s with no long arm material appear not to exist, presumably because of the lack of

the XIST locus and their consequent failure to inactivate (Dalton et al, 1998). Therefore, two cases with suspected i(Xp) (cases #12 and #13) in the present study were investigated by FISH technique in order to identify the status of the presumed isochromosome. Case # 12 was diagnosed by conventional cytogenetics as 46,X,i(Xp). Reassessment of this case by FISH analysis using FRAXA/E (Xq27-28), DXZ1 (centromeric), XIST (Xq13) and DMD (Xp21) probes confirmed the presence of a monocentric chromosome containing two short arms (one copy of DMD locus was present on each arm of the abnormal X). The long arm material (Xq27-28) was absent. Unfortunately, the XIST probe gave an inconclusive result. DXZ1 and XIST probes application showed a big fused signal on the abnormal X chromosome, which has not been seen in any other cases of the present study. The old condensed chromosomes of this case might be the cause of the fusion of the signals of the DXZ1 and XIST probes in such case. Therefore, the diagnosis t(Xp;Xp) has been raised. Late replication study could not be performed due to the lack of blood sample and the application of other probes specific for Xp and Xq was also not possible.

On the other hand, case # 13 was identified by G-banding as 46,XX/46,X?i(Xp). FISH study using different X-specific probes demonstrated the abnormal chromosome to be a derivative X in the form of (X;X) translocation. Hence, FISH has provided the final diagnosis and identified the breakpoints as t(Xp22;Xq22).

In the mean time G-banding for case # 14 showed i(Xq)s (defined as monocentric). It is difficult to distinguish between mono- and dicentric isochromosomes by GTG-banding due to the fact that one centromere in a dicentric is often inactivated which means that its site is not marked by a constriction (Fernandez et al, 1996). This limitation has been overcome by the use of the DXZ1 X-specific centromere probe in FISH technique, which gave two copies of the DXZ1 in the i(Xq) cell lines. This isodicentric chromosome is shown to have two copies of the q arm and two centromeres located very close to each other. The idic(Xq) generated complex mosaics of three cell lines. Isochromosomes for the long arm of the X chromosome are the most common structural abnormalities of the X being found in 15% of Turner syndrome patients (Hook and Warburton, 1983; Fernandez et al, 1996; Fernandez and Pasaro, 1998). Isochromosomes can originate either in meiosis or in mitosis. Wolff et al (1996)

suggested that the majority of i(Xq)s arise not by centromere misdivision but via breakage and reunion of susceptible regions of the short arm. The resulting i(Xq)s would be dicentric, leaving unstable acentric Xp fragments to disperse. The absence of a detectable 46,XX cell line indicates the meiotic origin of these chromosomes. Dicentric X chromosome of different derivatives were reported using FISH by Fernandez and Pasaro (1998). De La Chapelle and Stenstrand (1974) first suggested that some isochromosomes might be mitotically unstable and therefore give rise to a 45,X cell line. The degree of instability would depend on the amount of material between centromeres, so that a stable i(Xq) would be possible only when the centromeres are very close and acting in coordination or when, if relatively distant, one is rendered effectively inactive. Wolff et al (1996) observed in i(Xq) a direct correlation between the distance between centromeres and the presence of 45,X cell line, suggesting that a structurally dicentric i(X) might originally include two functionally active centromeres. These can form an anaphase bridge causing the isochromosome to break during early division of the zygote, thus giving rise to a 45,X cell line. The remaining cells, which still contain the isochromosome, would survive only if one of the centromeres become inactive, thus conferring stability (James et al, 1997; Dalton et al, 1998). Moreover, application of Y centromeric and PHY2.1 (Yq12) probes failed to detect any Y sequences in this patient although PCR analysis of her genomic DNA revealed the presence of Yq sequences (DYZ1 and DYZ2). Although FISH technique can detect low grade mosaicism, this requires scoring large numbers of cells. In such situation PCR analysis is widely used for detection of cryptic mosaicism of Y material (Chu et al, 1995, Osipava et al, 1998). It has been reported that 4%-30% of Turner patients are positive for Y material. Thus it is highly recommended to screen Turner patients by FISH as well as by PCR in order to detect hidden Y chromosome mosaicism (Chu et al, 1995; Lopez et al, 1998; Patsalis et al, 1998; Robinson et al, 1999).

The patient # 20 presented with multiple congenital anomalies and proved to have 46,XXp+ karyotype by G-banding technique. Detailed FISH analyses using a panel of X-specific probes and whole chromosome libraries of all chromosomes revealed that the extra material on the abnormal Xp was derived from chromosome 16. This finding was reconfirmed by using chromosome 16 arm paints (CAP 16p and 16q) which revealed that the Xp+ segment is of 16q

origin. Therefore, the comprehensive FISH analysis has detected the cryptic unbalanced translocation X:16 in this patient, leading to trisomy 16q and Xp monosomy. FISH analysis enabled the precise delineation of the karyotype in this case, which was not diagnosed by routine cytogenetic methods and the final karyotype was interpreted as 46,X,-X,+t(X:16)(p22.3;q24).

The abnormal phenotype in this patient could be due to partial trisomy 16q(q24→qter) or monosomy for Xp22. Late replication analysis confirmed that the der(X) was inactive in all the 50 cells scored. In females with balanced X/autosome translocation a preferential inactivation of the normal X in all the cells is exclusive. In case # 20, the der(X) due to t(X;16)(p22;q24) was confirmed to be inactive. However, the translocated segment 16q24→qter showed differential replication pattern. Thus the minor anomalies in the index case could be attributed to partial trisomy 16q as a result of unbalanced translocation. Parental karyotypes were normal.

The occurrence of balanced/unbalanced structural rearrangements involving an X chromosome and an autosome is very rare. They are associated with normal to severe pathogenesis, depending on the chromosomes involved and the extent of monosomy/trisomy of the segments involved. Inactivation and spreading effect may also contribute to the severity of the phenotype and pathogenesis. In human X/autosome translocations have been reported for all autosomes (1-22), The most frequently involved autosomes are 15, 21 and 22 (Schmidt and DuSart 1992; Borgaonkar 1997). The clinical significance of inactivation pattern in X/autosome translocations has been extensively reviewed by Schmidt and DuSart (1992). Unbalanced t(X;16) has not been reported previously. Davies et al (1976) have reported a case of familial balanced t(X;16)(p11;q24) in two normal female sibs inherited from the mother. The der X was confirmed to be late replicating in 100% of the cells. Partial trisomies 16q have been reported in many cases compatible with survival up to a year (Savary et al 1991). Most of the cases reported are de novo mutations, except for the case reported by Hirai et al (1981), resulting from unbalanced segregation of a parental translocation. Savary et al (1991) reported a case of trisomy 16q23→qter resulting from maternal balanced t(13;16)(p12;q23). However the trisomy16q phenotype has not been delineated as a clinical entity due to concomitant occurrence of

partial monosomy/trisomy of different autosome involved. The smallest segment of 16q trisomy reported by Savary et al (1991) presented with low birth weight, hypotonia, failure to thrive, and abnormal shape of the skull and high prominent forehead.

Normal male development depends on the presence of Y chromosome in human and other mammalian species. This chromosome is involved in numerous structural abnormalities (Hsu, 1994), and the phenotype of individuals with Y chromosome abnormalities depends on the deleted genes.

One of the important aspects of application of FISH in screening patients with Turner syndrome mosaics is to look carefully for the presence of a 46,XY or 46,X,+mar (? Y) cell line as these females have an increased risk (10%-20%) for the development of gonadoblastoma (Page D, 1987). The possibility of XY or marker chromosome (? der Y) should be suspected if the phenotype presents with ambiguous genitalia and mild stigmata of Turner syndrome. Here, two cases (cases # 17 and 18) presented with failure to thrive and developmental delay, and their karyotype was interpreted as mos 45,X/46,XY in the ratio 48:52 and 42:58 respectively. Application of FISH using X and Y specific probes confirmed the mosaicism in both cases. In one study, either an entire Y chromosome or a portion of it (e.g., marker derived from a Y chromosome) was present in 90% of individuals with dysgenetic gonads who develop germ cell tumors (Toeche and Hernandez, 1986). Other recent studies have implied that genes within the pericentromeric region of the Y chromosome are important in the causation of gonadoblastoma. Tsuchiya et al (1995) implicated a 4Mb region proximal to the centromere, while Salo et al (1995) implicated a 1-2 Mb region just distal to the centromere. These studies suggest that any female with a Y chromosome (or partial Y) containing these regions would be at an increased risk for the development of a gonadoblastoma (Schwartz et al 1997).

In the present study two cases (# 19 and # 23) interpreted by conventional cytogenetics using G-banding as having 45,X/46,X,+mar and 46,XY/46,X,+mar (?Y) karyotypes respectively were investigated. Reassessment of the karyotypes by applying FISH has delineated the marker chromosome in case 19 as isodict(X;Y). The detection of Y material in this patient has an important implication for increased risk of gonadoblastoma development. In the second case (# 23), FISH identified the 46,XY cell line in 42 cells examined (84%), 5 cells (10%) were

demonstrated by using different Y-specific probes to be i(Yq) while r(Y) was detected in 3 cells (6%). Application of FISH technique had delineated the presence of a third cell line. Thus these findings demonstrate successfully the value and necessity of FISH utilizing DNA probes in the identification of sex chromosome markers.

Two females with primary amenorrhea have been ascertained by FISH analysis to have structural Y chromosome abnormalities with absence of the *SRY* sequences. Application of FISH using a panel of X and Y specific probes in case # 21 who presented with the karyotype of 46,X,t(Xp:Yq) by GTG-banding had precisely delineated the breakpoints in this case. Her final karyotype was interpreted as 46,X,der(X)t(X;Y)(Xqter→Xp22::Yq11→Yqter). This is the most common type of X/Y translocation. Twenty-five of such cases have been reported and reviewed by Hsu (1994). In this type of translocation there is a normal X chromosome and the second X chromosome is a derivative X with a portion of Y (Yq11 to Yq12) translocated onto the distal short arm (Xp22). All the cases reported were females. Because of the deletion of the distal short arm of the X including the telomere (monosomy for Xp22.3), many of these patients were short with short limbs. Most patients were either proven to be fertile or said to have normal ovaries. Some had mental retardation (Hsu, 1994). Application of FISH analysis on the second case #22 confirmed her karyotype as 45,X/46,Xi(Yq). The i(Yq) was found to be monocentric. Fisher et al (1990) proposed that the haplo-insufficiency of a gene (ribosomal protein gene, *RPS4Y*) mapped on Yp contributes to the Turner phenotype and concluded that one or more Turner genes are probably located in the interval 1A1. The almost exclusively monocentric Yq isochromosomes may arise because inactivation of one of the centromeres renders stability to the chromosomes and prevents their loss at anaphase.

The basic sex determining factor responsible for switching on male development is known as the *SRY* gene, located at Yp11.2 (Sinclair et al, 1990). Cases # 19 and #21 in the present study were females. No *SRY* gene was found in those two cases and thus their female phenotype might be attributed to the absence of a regulatory *SRY* gene product. Individuals with no Yp and therefore lacking *SRY* are all phenotypic females with sexual infantilism and streak gonads (Hsu, 1994).

Several previous studies have used PCR and PCR-Southern blot analysis to detect the presence of SRY and/or other Y specific loci in Turner syndrome patients after conventional cytogenetic technique failed to identify a Y chromosome cell line in their karyotype (Binder et al, 1995; Henegariu et al, 1998; Lopez et al 1998). Recently Kocova et al (1993) and Chu et al (1995) have shown that when Y-specific PCR is combined with Southern blotting, 4%-30% of 45,X patients are positive for some part of the Y chromosome. Medelj et al (1992) detected SRY gene in 1 (2.5%) of 40 patients using PCR amplification. This low prevalence identified in a low level of Y mosaicism was confirmed by Binder et al (1995) and Patsalis et al (1998).

4.2 Molecular studies

4.2.1 Mutational analysis of the AR gene

Androgen insensitivity syndromes (AIS) are the clinical manifestations of end-organ resistance to androgen actions. In this inborn error of sexual differentiation, the most common cause of end-organ resistance is altered androgen receptor (AR) function. It is an X-linked disorder in 46,XY subjects in whom normal male sexual differentiation in utero is altered and subsequent neonatal and pubertal virilization is absent or impaired (Brown 1995; Quigley et al 1995). The mechanism of action of the androgen receptor involves binding of the ligand, which promotes conformational changes to overcome the inhibition to dimerization. The receptor is translocated to the nucleus then it binds to a specific region of DNA, the hormone response element, eliciting its actions within the target cells (Siegel SF, 1995).

Many qualitative and quantitative AR abnormalities, causing a broad range of AIS phenotypes, have been described (Gottlieb et al 1996). The spectrum of phenotypes ranges from individuals with completely female external genitalia and absence of Mullerian and Wolffian duct derivatives (complete AIS) to patients with ambiguous genitalia or with mild hypospadias (partial AIS) (De Bellis et al 1994; Quigley et al 1995). Infertility in phenotypically normal men has also been reported as a manifestation of androgen insensitivity (Wang et al 1998). Quantitative and qualitative alterations in androgen receptor binding activity can usually be demonstrated in cultured genital skin fibroblasts from affected subjects.

More recently, the molecular lesions responsible from AIS have been identified as a variety of mutations in the AR gene (Batch et al 1992; McPhaul et al 1993). The greatest numbers of different mutations so far identified are located within exons encoding the steroid-binding domain properties (Brown et al 1994; Murono et al 1995).

The androgen receptor is encoded by a single gene containing eight exons located on the Xq11-12 (Lubahn et al 1989). Like the other steroid hormone receptors, the AR consists of distinct functional domains. The N-terminal part is involved in transcription activation and is encoded by exon 1 (Faber et al 1991). Two highly conserved DNA-binding zinc clusters recognizes specific consequences, whereas the C-terminal zinc cluster is involved in dimerization (Jenster et al 1991; Luisi et al 1991). Parts of exon 3 and 4 encode the hinge region, which contains a nuclear import, and exons 4-8 encode the ligand-binding domain (McPhaul et al 1993).

The patients investigated in the present study constituted a heterogeneous group according to their clinical phenotype. Two individuals presented with classical features of testicular feminisation (complete AIS: CAIS), while three patients had a predominantly male phenotype and clinical findings consisted of hypospadias and/or micropenis (partial AIS: PAIS). The coding regions of the gene were amplified by the polymerase chain reaction and screened for mutations by means of SSCP and HA. Then was followed by DNA sequencing of putative mutant segments.

Initially, cytogenetic and molecular studies showed no deletion in the Y chromosome or in the coding region of the *AR* gene in all investigated patients. Around 5-10% of the *AR* gene mutations causing AIS are due to deletions. Complete *AR* gene deletion in two families and partial deletions involving multiple exons (4-8, 3-8, and 6-7) and single exons (2, 3, or 5) have been reported for families with complete AIS (Brown TR, 1995).

PCR-SSCP analysis in the present study revealed an abnormal migration in a 347 base pair fragment amplifying exon 8 of the AR gene in one patient (Fig 3.40). This substitution resulted from point mutation at nucleotide 4109 G → T transversion causes Val 889 Leu. Screening 100 chromosomes from unrelated individuals revealed absence of this base pair substitution indicating that it was not a polymorphism. This and other studies (Batch et al 1993; De Bellis et al

1994; Quigley et al 1995; Gottlieb et al 1996; Bruggenwirth et al 1997) indicate that complete as well as partial AIS could arise from altered function of the DNA and steroid-binding domains of the AR. The possibility of associated mutations in noncoding regions of the AR gene has not been excluded completely.

In the present study conversion of valine to leucine at amino acid 889 resulted from a single base mutation in exon 8 was associated with complete AIS. Previous studies of alternative amino acid substitutions at the same given codon Val 889 in the *AR* gene were also reported in CAIS subjects (Pinsky et al 1992; De Bellis et al 1994). Valine at codon 889 was replaced by methionine in two unrelated subjects with complete AIS. Conversion of valine to leucine was reported in exon 7 of the *AR* gene four times in AIS patients (Gottlieb et al, 1996). Both valine and leucine are non-polar amino acids. However, there are known examples of conservative missense mutations that can results in a disease phenotype if they occupy sites in the protein that are key determinants of stability or function (Pinskey at al, 1992; De Bellis et al, 1994; Gottlieb et al, 1996).

The valine 889 by leucine substitution, which lies in the ligand-binding domain of the receptor within the conserved region, postulated to function as a dimerization domain (Fawell et al 1990). This is a relatively conservative amino acid change and even though the mutation was not detected in any of the other 4 patients or 100 normal males screened, it could not formally discount the possibility that it is a polymorphism, with no effect on receptor function. Functional studies of an androgen receptor containing this mutation will provide an unequivocal resolution of this uncertainty. However, this effect of Val 889 replacement by Leu alone could be sufficient to cause the complete lack of masculinization during fetal development. The mutation also created a restriction site for *Alu1*, which could allow the carrier status of female(s) to be determined, and a prenatal diagnosis to be performed. This mutation in the steroid-binding domain provided more insight into the mechanisms of receptor function and could attribute also to the growing database of the structure function map of the *AR* gene.

Great numbers of different mutations so far identified are located within exons encoding the steroid-binding domain properties (Muroso et al 1995; Gottlieb et al 1996). Relatively few mutations occur within the most C-terminal region of the steroid-binding domain encoded by exon 8. However, the occurrence

of AIS related to these mutations demonstrates the critical nature of this region for maintenance of steroid binding and transcriptional activity of *AR* (Brown TR, 1995).

Mutations at several different sites in exons 2-8 encoding the DNA and androgen-binding domains with a relatively high number of mutations in two clusters in exons 5 and 7 have been recently been identified in *AR* gene in subjects with both complete and partial androgen insensitivity (Evans et al 1996). The number of mutations in exon one are extremely low (Batch et al 1993; Brown TR, 1995; Gottlieb et al 1996; 1997). CAIS is most often attributed to point mutations in the *AR* gene causing amino acid substitutions, aberrant splicing, or premature termination (Shkolny et al 1995; Bruggenwirth et al 1997). The evidence to date indicates that a mutation of the *AR* gene is responsible for most if not all cases of CAIS (70%) (Emery and Muller 1992). In contrast, many patients with PAIS demonstrate no defect in the androgen binding and no *AR* gene mutation could be identified in them (Batch et al 1992; McPhaul et al 1993). In such cases, it must be assumed that partial androgen insensitivity is the result of defects in other genes, whose expression is controlled by the androgen receptor gene during genital development (Batch et al 1992; Viner et al 1996).

Recently, a database of the *AR* gene mutation has been established and described by Gottlieb et al 1996. It shows the androgen-binding phenotype of the mutant *AR*, the clinical phenotype of the affected persons, the family history and whether the pathogenicity of a mutation has been proven. The database has allowed substantiating and amplifying the observation of mutational hot spots within exon encoding the *AR* binding (exon 4-8).

Molecular genetic analysis for diagnostic purposes employing SSCP has proven to be a very useful approach to a variety of diseases (Orita et al 1989). It has been used with success by several groups for characterization of point mutations in the *AR* gene (Hiort et al 1994; Bruggenwirth et al 1997; Wang et al 1998). SSCP has the advantage that laborious sequencing reaction can be limited to the very few samples that have shown a different migration pattern on the gel electrophoresis. Using PCR-SSCP analysis, three cases with PAIS and one case with CAIS in the present study were found to have no mutation in the coding region of the *AR* gene. The present study and other reported ones showed that in

some individuals, clinically diagnosed as having AIS, no mutation was found in the coding region and exon-flanking intronic sequences of the *AR* gene (Bruggenwirth et al 1996; Weidemann et al 1996) and DNA sequencing (Morel et al 1994), in spite of clear phenotypic, endocrinological, and biochemical evidence for AIS. Mutations might be missed because PCR-SSCP is not 100% sensitive. Often, only the exonic sequences and their flanking intronic regions are screened, leaving mutations in important intronic and promoter regions undetected. Recently, Bruggenwirth et al (1997) reported intronic mutation in a family with PAIS. By sequencing part of intron 2, a T→A mutation was found 11 bp from exon 3. Furthermore, mRNA analysis of the same family, revealed that splicing involved a cryptic splice site, located 71/70bp upstream of exon 3, resulting in generating mRNA with an insert of 69 nucleotide. For the *AR* gene, if cells from patients are available, RT-PCR studies and western immunoblotting can be very informative, in particular when mutations are present in intronic regions (Ris-Stalpers et al 1990; 1994). Expression of a number of genes involved in male sex differentiation and development is regulated by the androgen receptor (AR).

There are other explanations for the apparent absence of AR mutations in several AIS cases. Neutral mutations for example have to be interpreted with caution. Ritchard and Beckmann (1995) have found a 'neutral' point mutation in the cDNA of the calpain (CANP3) gene, which turned out to be pathogenic, because a splice donor site was created. Kallio et al (1996) suggested that, in 46,XY subjects without a mutation on the *AR* gene and with a typical AIS phenotype, post receptor defects might be the cause of the disease. This may involve receptor-specific cofactors or corepressors. Recently, a family with dominant inheritance of thyroid-hormone resistance was reported, which could not be linked to defects in the thyroid hormone-receptor alpha or beta gene (Weiss et al 1996). It was postulated that an abnormal cofactor, playing a role in regulation of thyroid-hormone action, might be involved. Most cofactors reported to date are not AR specific, so mutations in these factors will probably be lethal or give rise to a complex phenotype. Yeh and Chang (1996) have reported one coactivator, ARA70, which binds specifically to the ligand-bound AR. It is not known whether mutations in the gene encoding ARA70 correlate with certain forms of AIS (Bruggenwirth et al 1997).

Although CAIS, is not life threatening, affected individuals are infertile and require counseling, gonadectomy, hormone therapy, and sometimes vaginoplasty (Davies et al 1995). Many families therefore request genetic counseling. On the other hand, PAIS is a much more variable condition, which present with an intersex phenotype at birth. Therefore, management of PAIS requires a decision regarding sex rearing based on the genital phenotype, genital reconstruction surgery and hormone therapy.

Once a mutation is found, it may be used to provide information for genetic counseling of other family members. Alternatively, the intragenic trinucleotide repeats in the N-terminal region of the receptor may be used as polymorphisms to follow the affected X chromosome through the family (Davies et al 1995).

4.2.2 Mutational analysis of *SRY* gene in sex reversed patients

Sex differentiation is a complex physiologic process that most likely involves the products of many genes, not only on the Y chromosome, but also several that are X-linked and autosomal. For an infant to develop as phenotypically complete male or female a cascade of complex molecular and morphologic events must occur at the appropriate time and in the correct sequence during ontogeny. Normal human sex differentiation consists of three basic steps (Jost A, 1972). Each of these steps involves a number of complex, sequential processes. The first step is the establishment of genetic sex, which is determined at fertilization. In the second step an indifferent gonad differentiates into a testis in the XY male or an ovary in the XX female. In the absence of they chromosome, a tests-determining pathway fails to be initiated or is blocked, and development of the fetal gonads follows an inherent ovarian pathway. Testis development from the embryonic bipotential gonad depends on the inheritance of a Y chromosome-encoded gene, which was previously referred to as the testis determining factor (TDF) and subsequently identified as the *SRY* (sex-determining region Y) gene (Sinclair et al, 1990). *SRY* most likely activates a succession of other genes which switch the inherently female pattern of development to that of the male, beginning with testis formation. The final step in the pathway of normal sexual differentiation, the translation of gonadal sex into phenotypic sex, depends on the type of gonad formed. Indifferent internal and

external genitalia precursors are converted to male or female structure, depending on whether or not a testis develops (with notable exceptions exemplified by the testicular feminization syndrome). Hormones produced by the fetal testis are responsible for the induction of masculinization of the external genitalia and development of the structures of the male reproductive tract, as well as prevention of the development of the uterus and fallopian tubes (Angle et al, 1997).

The investigation of subjects with sex reversal has been a central strategy in the study of mechanisms responsible for normal gonadal and genital differentiation. Information from the study of 2 types of sex reversal was used to map the locus of the testis-determining factor (TDF) gene to the distal short arm of the Y chromosome (Vergnaud et al, 1986). One type included the so-called 46,XY females. The other included subjects with 46,XX sex reversal (Turner et al, 1995).

4.2.2.1 XY females

Studies in the past few years have clearly established the *SRY* gene (sex determining region, Y) to be the primary testis-determining gene in humans (Sinclair et al, 1990; Koopman et al, 1990). The *SRY* gene is located on the short arm of the Y chromosome and consists of a single exon. Analysis of the *SRY* open reading frame (ORF) has identified a domain termed the HMG box, which is conserved with regard to a number of proteins associated with DNA, including the high mobility group (HMG) nuclear proteins (Gubby et al, 1990; Sincalir et al, 1990) and various transcriptional factors, such as T cell factor 1 and lymphoid enhancer factor 1 (Ferrari et al, 1992). Mutations in the *SRY* gene can cause a failure of testicular development that may result in complete or partial male to female sex reversal. In some cases, these mutations have been demonstrated to alter the DNA-binding and/or bending properties of the *SRY* protein (Pontiggia et al, 1994; Veitia et al, 1997).

The term 46,XY gonadal dysgenesis has been used to describe various conditions of abnormal gonadal differentiation. The 46,XY complete gonadal dysgenesis is characterized by absence of testes, affected subjects presenting with female genitalia, normal Mullerian structures and streak gonads like those of Ulrich-Turner syndrome. This condition is also called 46,XY pure gonadal dysgenesis (Sohval AR, 1965). In some individuals, there is partial testis

determination and affected subjects present ambiguous genitalia of a mixed Mullerian and Wolffian structures and dysgenetic gonads. These cases are referred as 46,XY partial or incomplete gonadal dysgenesis (Berkovitz et al, 1992), although terms such as 46,XY dysgenetic male pseudohermaphroditism (Federman DD, 1967) have also been applied (Marcantonio et al, 1994).

Swyer (1955) was the first author to document cases of XY gonadal dysgenesis, and this condition was initially referred to as Swyer syndrome. Over 120 cases of gonadal dysgenesis have been reported (Bercu and Schulman 1980). Sporadic cases occur, but many have been familial, compatible with autosomal recessive inheritance limited to karyotypic males or X-linked recessive inheritance (Hersh et al, 1980; Phansey et al, 1980).

In the present study cytogenetic, molecular cytogenetic (FISH) and molecular studies of 4 subjects with 46,XY sex reversal were carried out to delineate their molecular pathology. Two cases presented as pure gonadal dysgenesis (complete gonadal dysgenesis and the other two presented with ambiguous genitalia and dysgenetic gonads (partial gonadal dysgenesis). Cytogenetics and molecular cytogenetics (FISH) using X and Y centromeric probes analyses performed on blood failed to detect 45,X and/or 46,XX cell lines in all investigated cases. In some patients, intersex abnormality is associated with sex chromosomal mosaicism, particular 45,X (Wolman et al, 1985; Liou et al, 1997). Thus, it is very unlikely that the abnormal sexual development in all patients is related to the presence of Y chromosome mosaicism. Yet, cytogenetic analysis of cells from both gonads was not studied and it needs to be investigated in all patients in order to exclude gonadal mosaicism. Moreover, in situ hybridisation to the XY female's chromosome using hocSRY/GMGY10 probes specific for SRY region (Figure 3.45) showed that the *SRY* gene was located on the short arm of their Y chromosomes. Therefore, the possibility of presence of microdeletion or Y chromosome rearrangement was also excluded in all 4 investigated patients. The pathogenesis of the XY gonadal dysgenesis phenotype appears to be heterogeneous, resulting from a variety of defects in the testis-determining/differentiation pathway. Some XY females with gonadal dysgenesis have lost the sex-determining region of the Y chromosome by terminal exchange

between of the sex chromosomes (Vergnaud et al 1986) or by other deletions (Davis RM, 1981; Page et al 1990).

A 790 bp PCR product of *SRY* open reading frame (ORF) encompassing the HMG-box was amplified from genomic DNA from 4 unrelated XY sex reversal subjects. The radiolabelled PCR products were analysed for mutations by CCM assays revealed none of the PCR products from the *SRY* open reading frame region showed aberrant CCM pattern. As confirmed in this study, the majority of XY females with gonadal dysgenesis show no evidence for alteration in the DNA-binding domain of the *SRY*. In some cases, sex reversal could result from mutations in regulatory regions of the *SRY* gene, which manifest itself immediately because of the hemizygous state of the Y chromosome (McElearvey et al, 1992b). In this study a 422 bp segment of the *SRY* promoter region was included in the SSCP analysis. Two overlapping PCR fragments, together spanning position -444 to -22 of the *SRY* promoter region (Behlke et al, 1993) and at position 429-650 of poly A tail, were amplified for DNA of the same patients also revealed that these two regions have no aberrant SSCP pattern in all investigated patients. Sequencing of the *SRY* ORF, promoter and poly A tail regions further, indicated all 4 patients harbor normal sequences. The absence of mutations in the *SRY* promoter region is consistent with a recent study that described a single strand conformation polymorphism screening of 360 bp including the minimal promoter, in a group of 45 patients in whom no sequence variants were detected (Schmitt-Ney et al, 1995). An independent analysis of 2 kb DNA immediately 5' to the *SRY* ORF of 49,XY females and XY intersex subjects detected two base changes; one was the common polymorphism and the other was unique but familial (Kwok et al, 1996). This suggests that mutations in the promoter regions essential for gene expression, although documented in a few cases, are rare events when compared with the frequency of mutation in coding regions (Koivisto et al, 1994). These results suggest that there may be other sequences crucial for the regulation of the *SRY* gene lying outside the regions studied or other sex reversing gene(s) able to produce indistinguishable phenotype.

To date, 24 mutations have been described in the coding region of the *SRY* gene, most of them being located in the HMG box. However, these mutations have been identified in only about 10-15% of XY females without a Y

chromosome deletion have mutation in the *SRY* gene (Berta et al, 1990; Hawkins et al, 1992; Schmitt-Ney et al, 1995; Kwok et al 1996; Veitia et al, 1997). The majority of mutations that have been tested are *de novo* and are not shared by the fathers of these XY daughters (McElreavey et al, 1996; McElreavey and Fellous, 1997; Brown et al, 1998), yet familial type of *SRY* mutations have been reported (Zeng et al, 1993; McElreavey et al, 1996). In other cases male to female sex reversal in the presence of a wild-type *SRY* ORF, the phenotype may result due to mutations in the regulatory region of the *SRY* gene (Schmitt-Ney et al, 1995), or because of mutations in other sex-determining genes may be located on the X chromosome or autosomes (Eicher and Washburn, 1986; Bennett et al, 1993; Foster et al, 1994).

To date, genetic approaches in sex reversed patients have identified several genes and regions involved in sex determination. An autosomal sex reversal locus, *SRA1*, has been identified in XY females with campomelic syndrome (Tommerup et al, 1993). Mutation in the Wilms tumor (*WT1*) suppressor gene in XY individuals reported to show abnormal development (Pelletier et al, 1991). Another autosomal sex reversing genes are located on distal 9p and distal 10q (Bennett et al, 1993; McElreavey and Fellous, 1997). A gene which regulates *SRY* has not yet been discovered. Regulation of *SRY* may involve the autosomal or X-linked genes described, or an unknown locus. Thus, XY gonadal dysgenesis is heterogeneous phenotype (Angle et al, 1997).

X-linked genes have been implicated in several familial cases of XY gonadal dysgenesis. Recently, a locus termed DSS (dosage sensitive sex reversal) has been defined, which consists of 160 Kb located at Xp21.3. Male to female sex reversal has been observed in individuals to be associated with duplications of this region in individuals with 46,XY karyotype (Arn et al, 1994; Bardoni et al, 1994). The severity of the abnormalities appears to depend upon the extent of the duplication. The DSS region at Xp21.3 was not screened for duplication in this study. Therefore it could not exclude the existence of a small duplication of this region. These results are in agreement with the previously reported studies (McElreavey and Fellous, 1997; Veitia et al, 1997). These cases may be candidate for harboring mutations at autosomal loci or X-linked. These results indicate that mutations involving the *SRY* gene on the Y chromosome are an infrequent cause of 46,XY gonadal dysgenesis.

4.2.2.2 XX males

The 46,XX male syndrome represents the most common condition in which the testes develop in the absence of a cytogenetically detectable Y chromosome. Most cases are sporadic. The incidence is estimated at about 1 in 20,000 newborn male infants (de la Chapelle A, 1981). As early as 1966, Ferguson-Smith suggested that the male phenotype in XX males arise as a result of unequal X-Y interchange during paternal meiosis.

During normal meiosis in male, crossover occurs between the tip of the short arm of the Y chromosome and the tip of the short arm of the X chromosome. These regions of the X and Y chromosomes contain highly similar DNA sequences. Because this crossover resembles the behavior of autosomes during meiosis, the distal portion of the short arm of the Y chromosome is known as the pseudoautosomal region. It spans approximately 2.5 Mb. Just centromeric to the pseudoautosomal region lies a gene known as sex determining region on the Y (*SRY*). This gene, which is expressed in embryonic development, encodes a product that interacts with other genes to initiate the development of the undifferentiated embryo into a male (e.g., Sertoli cell differentiation, secretion of mullerian inhibiting substance (Koopman et al, 1991). The *SRY* gene was mapped to a 35-kb segment on the distal region of Yp (Palmer et al, 1989; Sinclair et al, 1990).

The identification of *SRY* gene as the gene for TDF (Koopman et al, 1991) made it possible to confirm Ferguson-Smith's (1966) hypothesis by demonstrating that genomic DNA from most normal appearing subjects with 46,XX maleness contained sequences for *SRY*. By contrast, most 46,XX males with abnormal genitalia lack sequences for *SRY*. Similarly, almost all subjects with 46,XX true hermaphroditism lack sequences for *SRY* in genomic DNA (Ferguson-Smith et al, 1990; McElreavey et al 1993a; Turner et al, 1995).

In the present study cytogenetic analysis, fluorescent in situ hybridisation (FISH) and molecular amplification have been used to detect the presence or absence of Y sequences and to characterise the transfer of Yp fragments to Xp22.3 in 4 XX males. PCR amplification of the *SRY* gene, ZFY, PAR and Y1/Y2 sequences along the Y chromosome were applied to characterise the Y chromosome sequences present in 4 XX males. In three cases, PAR, *SRY*, and

ZFY sequences were amplified, while Y1/Y2 (centromeric sequences) failed to amplify (negative). Some authors have reported that the Y sequences present in the XX males extend from distal Yp to Y centromere, showing a variation in the size of the fragment exchanged from less than 40 kb (Sinclair et al, 1990) to more than 11Mb (Affara et al, 1986; Ferguson-Smith et al 1990). The similarity of the clinical features in all cases reported in the literature confirms that *SRY* gene is responsible for testicular determination, and that other genes on the Y chromosome are not so important in defining the phenotype. XX males which are *SRY* positive are the most frequent type of XX males reported (Affara et al, 1986; Page et al, 1987). This confirms the relative frequency of an Xp/Yp interchange originating through unequal crossing over between homologous pseudoautosomal regions in the paternal meiosis extending the exchange to sex specific sequences (Ferguson-Smith 1966; Weil et al, 1994). Some cases of Y;autosome translocation have been described in 45,X subjects with sex reversal (Munke et al, 1988). However, only one subject of 46,XX male with a Y;autosome translocation has been reported by Ester et al (1998).

In this study all patients had the Y chromosome DNA transferred to Xp22.3 as found in all other reported cases (Van der Auwera et al, 1992; Anderson et al, 1986; Magenis et al, 1987). The phenotype described in these cases is that of males with sterility owing to the absence of germinal cells and hypogonadism without sexual ambiguities, consistent with the clinical data detected in patients investigated in this study. The clinical features were very similar in those 3 cases, showing a male phenotype with small testes, testicular atrophy, and azoospermia. These patients had normal intelligence and a stature within normal male range.

Only one subject presented in this study had 46,XX sex reversal and absence of *SRY*. This was demonstrated by failure to detect *SRY* sequences in genomic DNA by PCR amplification and FISH analysis. In addition, all 4 subjects lacked sequences for Y centromere. Hidden mosaicism with a Y-bearing cell line was ruled out due to the absence of Y centromeric sequences by FISH analysis (Figure 3.51) using PDPY97 probe and by PCR using Y1/Y2 primer sets. Therefore, FISH analysis and PCR test confirmed this finding.

Direct detection of Y sequences in XX males has been previously shown to be possible by isotopic in situ hybridisation (Anderson et al, 1986; Magenis et

al, 1987; Schempp et al, 1989). However, the procedure and the interpretation of the results is facilitated in FISH (Fan et al, 1990). The first report in which translocation of an SRY-bearing fragment to an X chromosome in XX males could be directly demonstrated using FISH (Van der Auwera et al, 1992). Ester et al (1998) have used cytogenetic, FISH and PCR amplification to characterise the transfer of Yp fragments to Xp22.3 in six XX males and they have prenatally diagnosed XX male in the fetus with male genitalia detected by ultrasound and a 46,XX karyotype in amniocytes and fetal blood. Molecular analysis of fetal DNA showed the presence of the SRY gene. FISH techniques also showed Y chromosomal DNA on Xp22.3 in metaphase cells of a fetus. This is the second molecular prenatal diagnosis reported of an XX male but, the first one to be of a Y positive XX male, as the only other prenatal case reported was XX male fetus lacking any Y sequences (Vilain et al, 1994). The case of a prenatally diagnosed XX male is especially interesting as an example of the problems that may arise in a laboratory when the sex of a fetus is different from that expected based on prenatal cytogenetic analysis. FISH and molecular studies allowed the confirmation of the XX male diagnosis in the fetus. Other cases were detected only after the birth of a male following cytogenetic prenatal diagnosis of 46,XX (Sachs et al, 1992), and where postnatal allowed the possibility of a sample error or maternal cell contamination to be ruled out (Ester et al, 1998).

46,XX males can be classified as Y positive or Y negative, depending on the presence or absence of Y-specific sequences (Ferguson-Smith et al, 1990). In Y negative cases, autosomal or X-linked mutations may be responsible for testicular determination in the absence of SRY (de la Chapelle, 1987; Ferguson-Smith et al, 1990). Most 46,XX male patients with normal genitalia are SRY positive. In contrast, the majority of the XX males with genital ambiguity are SRY negative (Ferguson-Smith et al, 1990; McElreavey et al, 1993b). Nevertheless, exceptions do occur and Y negative XX males may exhibit complete masculinization (McElreavey et al, 1993b; Ramos et al, 1996; Zenteno et al, 1997) while genital ambiguity can be observed in Y-positive XX males (McElreavey et al, 1992a; McElreavey et al, 1993a; Lopez et al 1995).

The present study also confirmed the existence of one SRY negative XX male. This SRY negative XX male was also negative for all other Y sequences tested by FISH or PCR test. Other analysis using DNA amplification also revealed

some XX males who were Y-negative for several Y sequences, including ZFY (Oster and Clayton, 1989) or SRY (Ferguson-Smith et al, 1990) and excluding XX/XY or XX/der (Y) mosaics with an unprecedented sensitivity. The male phenotype in such cases might be explained by mutation (s) in one or more gene(s) downstream from *SRY* in the sex-determining pathway. The elucidation of the different steps and genes involved in the mechanism of testis developments may allow testing of this hypothesis. With respect to the phenotype, some authors have described abnormalities in the external genitalia (hypospadias, cryptorchidism) and a more prominent gynecomastia in Y negative XX males (Abbas et al, 1990; Ferguson-Smith et al, 1990).

XX-individuals lacking SRY who exhibit masculinization (complete or partial) are proposed to be defective in other genes and are able to express male sex determination in the absence of the master gene (*SRY*) of a regulatory cascade. On the contrary, XY sex reversal with an intact *SRY* gene can carry a mutation that renders another gene of the cascade insensitive to SRY action (McElreavey et al, 1993a). Many familial cases have been negative for Y chromosome-specific probes (Abbas et al 1990; McElreavey et al, 1993b). Pedigree analysis lead to the hypothesis that they carry recessive mutations (McElreavey et al 1993a).

Three clinical categories of sex-reversed 46,XX individuals have been identified: phenotypic males, male with ambiguity, and true hermaphrodites (de la Chapelle A, 1987). All patients are shorter than normal XY males, which is probably due to the absence of Yq-specific growth gene(s) (Ogata and Matsuo, 1992) and to the lack of an appropriate testosterone-dependent pubertal growth spurt (Boucekkine et al, 1994). Although all XX males have similar genotypes, different phenotypes have been described, suggesting that in the same cases *SRY* alone is adequate for complete male sexual differentiation while in others it is not sufficient for normal virilisation. In contrast, *SRY* negative males have ambiguous genitalia and bilateral testes or ovotestes, i.e.; they are true hermaphrodites (Lopez et al, 1995).

The availability of specific DNA probes for different regions of the Y chromosome demonstrated that approximately 80- 90% of XX males carry Y sequences due to an illegitimate X/Y interchange during paternal meiosis (Abbas et al, 1990; Ferguson-Smith et al, 1990; Weil et al, 1994). The rest who lack Y material their maleness is probably due to mutations in autosomal genes as in

campomelic dysplasia (17q) and WAGR syndrome (11p) and in 9p-, or in X-linked testis determining genes as in DSS gene (McElvery et al, 1993a, b; Wolf U, 1995; Ramos et al, 1996). Molecular studies in this study confirmed the absence of Y-derived material (PAR, SRY, ZFY, and Y1/Y2 centromeric) in one patient with ambiguous genitalia, as all the specific sequences analysed did not amplify, while they were present in male controls.

To explain the occurrence of Y-negative XX males, McElreavy et al (1993a) proposed that the *SRY* gene act by inhibiting a regulatory autosomal recessive gene, termed Z, whose product is a negative regulator of male sex determination. According to this model, XX individuals lacking *SRY* who exhibit a complete male phenotype would be homozygous for a Z null mutation. In contrast, XX males with genital ambiguity and XX true hermaphrodites may carry leaky Z mutations that conserve some residual activity, thus explaining the phenotypic variability. An alternative model suggested by Jimenez et al (1996) who proposed that X-linked locus, DSS located in Xp21 and subject to X inactivation, may act as a repressor of the male pathway. This implies that a single active copy of this gene can repress the male pathway in XX females, while in XY males it is inactivated by *SRY*, equating DSS to the hypothetical Z gene. According to this model, *SRY*-negative XX males with normal genitalia are homozygous for deletions or loss-of-function mutations in DSS, while *SRY*-negative XX males with genital ambiguities could be heterozygous for a DSS allele, such that the proportion of cells of the indifferent gonad in which the DSS + is inactivated would determine the degree of residual maleness. A gene isolated from the Xp21 region DAX1, has been proposed to be identical to DSS (Bardoni et al, 1994; Zenteno et al, 1997).

In summary, PCR amplification of *SRY*, *PAR*, *ZFY*, and *DXZ3* (Y1/Y2 centromeric) sequences could be effectively used in cases where cytogenetic analysis fails to detect the Y chromosome. PCR is more effective, and can provide detection of Y chromosome material in as few as 1 of 1,000 cells (Osipova et al, 1998). Further, it is more specific, and can be used for the precise detection of sequences responsible for the development of testicular tissue (*SRY*), or for regions implicated in the development of gonadoblastoma development (e.g., *AMGLY*). In this cohort, this approach considerably improved the diagnosis compared to classical cytogenetic analysis in 3 of 4 cases, and may be

recommended as an important tool in cases of cytogenetically undetectable markers or virilization of unknown origin.

In conclusion, the present study has shown that FISH is a valuable technique for the rapid detection of Y sequences in XX males. This technique provides direct evidence for the presence of Y sequences, including SRY, on the Xpter in XX males, and allows the detection of Y material with Yp-specific probes hocSRY and GMGY10 in metaphase and interphase nuclei of all Y positive males. Furthermore, PCR amplification using markers along the Y chromosome has allowed the Y sequences present in each patient to be defined and correlation with the phenotype to be established. Subsequently, the location of Y fragments has been further determined by in situ hybridisation on metaphase chromosomes.

Molecular and FISH techniques are very useful for detecting and locating Y sequences in cytogenetically XX males, allowing an accurate diagnosis and correct management of the patient. Testing new Y chromosome markers in XX males will make it possible to narrow the breakpoints further in each case and to establish correlation with the clinical features, identifying the Y region implicated in the definition of the phenotype (Ester et al, 1998).

4.2.3 Mutational analysis of male infertility

Between 2% and 12% of couples world wide are infertile, with the man responsible in approximately half of the cases (Cooke et al, 1998). Large proportions of these men are infertile either because of insufficient sperm (oligoospermia) or lack of sperm (azoospermia). Proposed causes of infertility in men include varicocele, obstruction of the spermatic ducts, agglutination of sperm, high semen viscosity, necrospermia, low volume of ejaculate, ejaculatory dysfunction, and high sperm density. When no cause is known, the man is described as having idiopathic infertility (Proyor et al, 1997). The cause of these defects in sperm production is unclear, but recent work points to both potential environmental and genetic causes.

In this study, 56 patients with either azoospermia or oligospermia were screened for the presence of mutation in the AZF region using 5-step strategies. To screen for large deletion and/or other structural rearrangements, all patients

included in this study were examined by (1) routine cytogenetic study, (2) multiplex PCR analysis, (3) Southern blot hybridisation and (4) FISH analysis with RBM cDNA probes. Lastly, to screen for point mutation and/or minor sequence alterations in the *RBM1* gene family, all patients were screened by SSCP, HA analyses and by DNA sequencing.

Cytogenetic study

Chromosome banding allows an analysis of the entire karyotype at one time, and is the preferred technique for routine diagnosis; therefore all patients were first analysed for chromosomal study in order to exclude the cytogenetically detectable chromosome abnormalities. Among the 56 men with infertility, the most common assigned diagnosis were idiopathic infertility with normal male karyotype (50 men or 89.2 %) (Table 3.5). Abnormal karyotype were found in 5 (8.9%) of the 56 subjects studied. Of these 5 subjects, 4 (7.1%) exhibited 47,XXY (80%) karyotype, the remained 1 (1.8%) had 47,XYY karyotype. Thirty-two of the men with infertility (57.1 %) had azoospermia, four had 47,XXY karyotype and one had evidence of spermatic duct obstruction. As 47,XXY karyotype is known to be associated with sterility, these patients were excluded from the molecular analysis. One azoospermic subject with 46,XY karyotype was also excluded from molecular study because of obstructive azoospermia. Twenty-four (42.9 %) had severe oligospermia, one of them had 47,XYY and varicocele. This case was also excluded from the molecular study because of the presence of varicocele. Thus, only 50 idiopathic infertile men with normal 46,XY karyotypes were further investigated by different molecular strategies in order to establish their pathogenesis.

Association between human male infertility and chromosomal anomalies has been known for a long time (Chandley A, 1979; Faed et al, 1979). The incidence of karyotype abnormalities among infertile men has been reported to range between 2.2 and 19.6% (Pandiyan et al, 1996; Gunduz et al, 1998; McLaren A, 1998). Thus, it would not be unusual to find chromosomal abnormalities in men attending infertility clinics. Therefore, karyotyping every male attending the infertility clinic would be necessary to identify or exclude those with genetic defects.

The exact mechanism by which chromosomal anomalies induce infertility is not clear. It is likely that the presence of abnormally distributed chromatin may interfere with meiotic division, therefore, reduces sperm production. Spermatozoa bearing abnormal chromosomes may cause abnormal embryonic development that can in turn cause early pregnancy loss (Evans et al, 1978). In the presence of the 47,XXY abnormality in Klinefelter syndrome, severe spermatogenesis failure causing a marked reduction in testicular size and azoospermia is the rule. Thus the reason for the patient's infertility is clear. In the 47,XYY abnormalities, however, testicular histology may be either normal or abnormal. Testicular size may be normal with only minor changes in the seminal profile (Pandiyan et al, 1996).

Multiplex and single PCR screening

Clinical experience indicates that large deletions of the Y chromosome, which can be seen under the microscope in late prophase and are detectable on routine karyotype, are uncommon in infertile men. However, submicroscopic deletions of the long arm of the Y chromosome, not detectable on karyotype and therefore called microdeletions, are present in 10-15% of azoospermic men (Vogt et al, 1993; Nagafuchi et al, 1993; Henegariu et al, 1994; Najmabadi et al, 1996). These microdeletions can be detected by polymerase chain reaction (PCR)-based sequence tagged sites (STS) mapping or by Southern hybridisation or by FISH analysis (Ma et al, 1992; Henegariu et al, 1994).

In the present study, molecular analysis of sequence tagged sites (STS) in azoospermic and oligospermic males was done. The screen was focused to Yq11; the region of genetic Y factors controlling spermatogenesis defined as AZF (i.e. azoospermia factor: Tieplo and Zuffardi, 1976). A rough molecular estimation of the length of the DNA region in Yq11, where AZF should be localised, indicates a minimum extension of 3-5MB (Vogt et al, 1993). Consequently, Y DNA probes used to screen for deletions of AZF gene sequences have to be spread along this Y region. However, a focus of the screening programme can be proximal and distal to Yq11, since microdeletions in this region have already been diagnosed in some azoospermic males (Vogt et al, 1992a; Nagafuchi et al, 1993; Henegariu et al, 1994; Kaboyashi et al, 1994). In a series of PCR experiments it can be shown that polymorphic amplification products are not visible and deletion breakpoints can be mapped reliably using the

maps of Ma et al (1992). Since this PCR Y analysis is possible without sophisticated blotting techniques and radio-labelled DNA probes, it became feasible to screen the Y chromosome of idiopathic sterile males in infertility clinics.

A total of 49 Y-specific STSs sets of primers covering Yq region were used in this study. 28 pair sets were used in 5 multiplex PCR reactions and the rest (21 pairs of primers) were used in single PCR reactions. Two pairs were *RBM* specific and two were within *DAZ* gene and one pair was *DAZ* gene specific. Most of STSs are located within Yq11 region.

Of the 50 men with idiopathic infertility only one azoospermic male (2%) was found to have a Y chromosome microdeletion. This approach showed, in one patient (case 25 in table 3.6), no amplification of six pairs PCR primers detected in 4 out 5 multiplex reactions. These deletions involved sY153 (subinterval C), sY147 and Y6HP52pr (subinterval D), sY 157 and sY 149 (subinterval E), and Fr15-11pr (not determined). No amplification failure was observed in 50 normal male controls including the father of the patient with microdeletions. The absence of an STS amplification product indicates the presence of a microdeletion in interval 6. Therefore, by molecular deletion analysis using multiplex PCR, the breakpoints in Yq11 was estimated to be in subinterval MX-MXII of the Ma map (Ma et al, 1992) between interval 6C and 6F according to Vollrath deletion map (1992).

Detailed deletion analysis using 21 STS primers within intervals 5 and 6 of Yq11 region showed that this patient had deletions in 16 more STSs. These refined physical map results confirmed the deletion between DYS232 and DYS239 which is localised to interval 6C and 6F on the distal part of Yq11 according to Vollrath et al (1992) deletion map.

Nearly all DNA loci listed in appendix II are single copy loci, which mean that their location in Yq11 is unique. It has been shown that lower repetitive DNA sequences are spread in Yq11 (Vogt et al, 1992b; Ma et al, 1992; Vollrath et al, 1992). A deletion of an Y-specific repetitive DNA locus can be detected by PCR analysis only if all sequence copies of this DNA locus are deleted. However, a critical analysis of PCR experiments should stress not only its higher sensitivity but also its sensibility for experimental artifact. It is highly recommended that the reproducibility of any deletion in the multiplex PCR experiment to be tested as

well in single PCR experiment using the corresponding primer since false deletion events are possible (Henegariu et al, 1994). This was the case when a few false deletions were detected by multiplex PCR, but the corresponding primer(s) was extended during a single PCR experiment.

Great interest has been devoted in the last several years to the identification of the gene(s) within the Y chromosome which are involved in the etiology of idiopathic male infertility (Vogt et al, 1992b; Nagafuchi et al, 1993). Two Y-specific candidate gene families have been cloned by deletion mapping of infertile men with Yq11.23 (interval 6) deletions and proposed as candidates for the putative AZF locus, the RNA-binding motif (RBM)-containing gene family (Ma et al, 1993; Najmabadi et al, 1996), and the deleted-in-azoospermia (DAZ) gene family (Reijo et al, 1995). Both are multiple-copy gene families. The *RBM* gene family has more than 30 copies spread through the Y chromosome (Meschede and Horst, 1997). Most of the copies are located in deletion interval 6A and 6B (Bhasin et al, 1997). The DAZ gene family has 6-10 copies (Cooke et al, 1998) located in interval 6D and 6E. At least two members of the RBM gene family, RBM-1 and RBM-2 are expressed in the testis (Najmabadi et al, 1996). The presence of RBM in the predicted protein sequence suggests that these genes play a role in RNA processing. However, the precise role of the RBM protein(s) in germ cell development remains unclear. On the other hand, *DAZ* has a testis-specific pattern of expression and encodes RNA-binding protein of yet undetermined function (Reijo et al, 1995). In the present study, using RBM1 and RBM2 specific primer pairs revealed that all patients included the patient with Yq11 microdeletions have retained RBM gene family. This could mean that RBM1 is not the only gene involved in the failure of spermatogenesis. In addition, because of the multigenic and dispersed nature of RBM sequence, only very large deletions would be expected to remove the entire possible PCR target.

Using *DAZ* specific primer pairs revealed that all patients and controls retained the *DAZ* gene, yet it was deleted in the patient with microdeletion. The father of the patient retained *DAZ* gene. Therefore, the patient in this study retained the *RBM1* and *RBM2* genes but has a deletion of the *DAZ* gene. The role of the *RBM* genes in male infertility has been challenged by the observations that many infertile men with Y deletions have had *RBM* sequence present. In fact, deletions of interval 6 have been detected in infertile subjects retaining *RBM*

(Kaboyashi et al, 1994; Reijo et al, 1995; Stuppia et al, 1996b). Moreover, deletions of *DAZ* have been identified in patients with different spermatogenic disorders (Reijo et al, 1995; Vogt et al, 1995). Although deletions involving the *DAZ* gene(s) appear to be the most frequent, several groups have described Y deletions that are outside the DAZ regions (Vogt et al, 1996; Pryor et al, 1997), which suggests that AZF could be owing to more genes (Reijo et al, 1995; 1996). Furthermore, only 10-15% of infertile men have Y deletions. These data suggest that additional Y-specific and/or autosomal genes may be involved in the infertility phenotype. Some observations suggest that *RBM* and *DAZ* are not the only genes responsible for idiopathic infertility (Meschede and Horst, 1997).

Microdeletions in Yq11 were reported in up to 10% of idiopathic sterile males (Ma et al, 1992; Vogt et al, 1993) while in the present study only 2 % of idiopathic sterile men showed microdeletion. When the PCR experiment fails to detect a deletion event in the Y chromosome of sterile males, different explanations are possible. First, the Y chromosome of the patient is normal with no deletion of AZF gene sequences in Yq11. Second, mutation in the gene structure of AZF can not be detected because they are either point mutations or very small deletions not hit by current set of screening probes. Clearly, analysis of deletions in Yq11 generally gives only an indication of the deletion of the AZF gene sequences as long as the gene structure of AZF is not known at the molecular level. Therefore, the PCR-Y-screening programs can hint at the deletion of AZF gene sequences but can not yet prove that this occurs. It is essential to prove that the extension of any DNA deletion detected on the Y chromosome by multiplex PCR analysis is restricted to the patient's Y chromosome and is not present as a rare polymorphic event in the male of the patient's family (Henegariu et al, 1994). Interestingly, the microdeletion detected in one patient was not detected in the rest of patients' investigated and male controls including the father of the patient with microdeletion. Therefore, this deletion probably includes at least part of the AZF gene structure. Moreover, clinical diagnostic tools for selecting patients with a potential disruption of AZF gene sequences out of the large idiopathic patient group are still very crude (Vogt et al, 1992a; 1992b). Microdeletions in Yq11 have been found in azoospermic as well as in severe oligozoospermic males (sperm count between $1-5 \times 10^6$ per ml). The patient's hormone level of LH and testosterone is usually normal. The level

of FSH is normal or increased within a broad range (Vogt et al, 1992a; Henegariu et al, 1994).

Recently, the rate of reported microdeletion ranges from 3-18% (Vogt et al, 1996; Meschede and Horst, 1997). The variability is most likely explained by different selection criteria for the study subjects. Most, but not all, Yq microdeletions described so far includes the DAZ gene cluster (Qureshi et al, 1996; Vogt et al, 1996). This has led Vogt et al (1996) to postulate the existence of three non-overlapping regions containing different azoospermia factors, termed AZFa, AZFb and AZFc. On the basis of the testicular histology in these men, the deletion of AZFa was associated with the presence of only Sertoli cells. The deletion of AZFb was associated with the developmental arrest of germ cells at the pachytene stage, and the deletion of AZFc with the developmental arrest of germ cells at the spermatid cells (Vogt et al, 1995). The AZFc coincides with the DAZ locus, but no candidate genes for AZFa and AZFb have been designated yet. Vogt (1996) also postulated that the different categories of Yq microdeletions might be specifically associated with particular types of testicular histopathology. However, studies by other groups have not confirmed this claim (Reijo et al, 1995; Qureshi et al, 1996; Meschede and Horst, 1997).

In this study the ultrasound examination in the patient with the microdeletion of interval 6 displayed normal testes without any appearance of obstruction. The levels of FSH, LH, and testosterone were within normal range and it seems that this patient has a deletion in AZFc associated with spermatogenic arrest.

In infertile men with DAZ deletions, both meiotic arrest and the Sertoli-cell-only phenotypes have been described (Reijo et al, 1995). It is possible that germ cell degeneration may occur secondarily. Testicular biopsies often reveal some tubules that show meiotic arrest and other tubules with the Sertoli-cell-only phenotype. It remains unclear whether these two histological phenotypes represent two ends of the same spectrum or whether they are the result of distinct genotypes.

Several investigators have shown that some infertile men carry submicroscopic deletions in Yq that are not present in their fathers' or brothers' Y chromosome (Ma et al, 1993; Kaboyashi et al, 1994; Reijo et al, 1995; Qureshi et al, 1996; Vogt et al, 1996). However, Stuppia et al (1996b) showed both a

proband and his father with a deletion in Yq11, but the deletion in the proband was actually larger compared with that in his father as the proband's deletion also involved STSs sY243 and sY 269 which are located within the more proximal subinterval E of interval 6. They confirmed the critical role of interval 6 in the spermatogenic process, which suggests that deletions of subinterval F, per se, is not associated with infertility. An additional point of interest is that both the proband and his father retained RBM1 and STSs sY254 and sY255, which are mapped within DAZ in subinterval D. The segment deleted in the proband only, which is likely related to infertility, lies within subinterval E, which is outside the DAZ region. They implied that, in oligospermic patients at least, other genes in the region distal to DAZ and RBM1 may be involved in the spermatogenesis process. Moreover, they argued that some deletions should not lead necessarily to infertility, but these deletions make the Y chromosome more liable to a second mutation resulting in spermatogenesis failure, as a consequences of DNA instability (Stuppia et al, 1996a).

The precise physiological function and role of RBM and DAZ gene families in human spermatogenesis remain unclear. The RNA molecules that are the targets of these RNA-binding proteins have not been identified. It is also not clear how deletions of one or two copies could explain infertility when there are multiple copies of these genes elsewhere on the Y chromosome.

On the basis of the infertility rate among men and the percentage of infertile patients showing deletions of the Y chromosome, Reijo et al (1995) have suggested that these mutations should occur in ~ 1 in 10^4 male newborns and that this high figure could arise by a mechanism involving repeated sequences flanking the gene.

Y-chromosome deletions are relatively frequent (10%) and their frequency increases with the severity of the spermatogenic defect. However, Y-chromosome microdeletions can not be predicted on the basis of clinical findings or even the result of semen analyses. The role of analyses of Y-chromosome microdeletions in evaluating men with infertility remains to be determined. With the advent of intra cytoplasmic sperm injection (ICSI) in the field of reproductive technology, the potential for passing on these defects to offspring is real and should be considered when infertile couples are counseled about this procedure ((Pryor et al, 1997). This raises issues of informed consent and ethical concerns.

Substantial prevalence of Y deletions in infertile men and the potential risk of transmitting this genetic disorder to their offspring provide a compelling rationale for screening of infertile men prior to ICSI. Considering the ease of PCR-based STS mapping, this screening could become readily available in clinical cytogenetic laboratories. The couples undergoing ICSI should be counselled about the potential risk of transmitting this genetic disorder to offspring. In addition, there is a pressing need for developing mechanisms for worldwide monitoring of ICSI babies for genetic disorders, including Y deletions (Bhasin et al, 1997).

Mutation detection in RBM1 gene

If *RBM* is involved in human spermatogenesis and altered in a proportion of infertile males, then, because it is a multicopy gene family on the human Y chromosome (Ma et al, 1993), it is possible that deletion or rearrangement would be a more common route to infertility than point mutation. However, if point mutations do play a part, a possible region for an effective single base change would be the RNA-binding domain of the protein, particularly in the RNP1 and RNP2 amino acid motifs (Prosser et al, 1996).

To rule out the possibility of structural rearrangement or deletion of the *RBM* gene family, all DNA samples from 50 infertile and 50 normal men were screened by means of FISH analysis and Southern blot hybridisation.

In situ hybridisation with MK5 and MK29 to metaphase spreads of infertile and normal males showed intense and multi-signals on both chromatids of the Y chromosome. Interphase *in situ* hybridisation also showed the presence of multiple clustered hybridisation signals. These findings confirmed that *RBM* gene family is multicopy as reported by Ma et al (1993). No deletion or structural rearrangement was detected in all cohorts including the patient who showed microdeletion in Yq11 upon multiplex PCR experiments. Because the *RBM1* sequences are known to be multigenic and dispersed throughout intervals 5 and 6, are likely to hinder detection of small deletion, large deletions are required to remove all specific *RBM* targets in order to be detected by FISH analysis (Ma et al, 1993).

DNA samples from 50 infertile and 50 normal men were digested with two restriction enzymes *EcoRI* and *TaqI*. In the present study the resulting hybridisation with MK5 pattern was the same. No deletion or structural rearrangement was detected in all investigated individuals. Molecular deletion

analysis with probes from interval 6 of the Y chromosome has found evidence for microdeletions in some azoospermic men (Vogt et al, 1992a; Ma et al, 1993). In this study Southern blot analysis failed to detect microdeletions in the RBM gene family confirming the FISH studies and multiplex PCR experiments.

In summary, FISH analysis and Southern blot hybridisation to DNA from 50 infertile men and 50 controls with cDNA encoding RBM1 and RBM2 respectively revealed the presence of multicopies of the gene and no deletion or structural rearrangements were detected in all investigated cases. These results are in agreement with the multiplex PCR studies. Therefore, the possibility that deletion or rearrangement of *RBM* gene family would be the cause of infertility is ruled out in the cases of the present work.

To provide proof that defects in a gene result in the disease for which it is a candidate remains problematic. One convincing route is to demonstrate a new point mutation in the gene in one or more patients with the disease. Therefore an attempt was done for the *RBM* gene family in a patient set (50 cases) with no visible chromosome deletions and with idiopathic defects in spermatogenesis. To search for mutations in the multicopy RBM genes that might be associated with male infertility, sequence data from the reported cDNA clones was used (Ma et al, 1993). This gene has 12 exons, three of which encode the putative RNA binding domain of the protein. Two particular sequence arrays in RBM are believed to be potentially important for its function (Ma et al, 1993). The RNA recognition motif (RRM) extends from amino acid 1 to 87 with the two evolutionarily highly conserved sequences, RNP1 in exons 3 and 2 respectively. (Prosser et al, 1996) The so-called SRGY (Serine-Arginine-Glycine-Tyrosine) box domain extends from amino acids 227 to 347 (exons 7-11) and may be characteristic of human RBM. It has been postulated that the SRGY box acts in protein-protein interaction related to gene function and specificity (Prosser et al, 1996).

With this in mind, the DNA of 50 patients with no known Y chromosome deletion was amplified with intronic primers specific for exons 1-12. Single strand conformation polymorphism (SSCP) analysis was optimised to screen exons 1-11 and three different copies of exon 2 (B, C, D), and heteroduplex analysis (HA) was optimised to screen exon 12 of the *RBM1* gene in all cohorts of the studied patients. For SSCP study, different running conditions were used. 0.5X MDE™

gel with 5% glycerol running at room temperature and at 4 °C were applied along the study. No band shift was detected upon analysis of all screened exons. However, in some instances, variant bands, which were interpreted as mobility shifts, were detected upon SSCP analysis of the PCR product from different exons of the *RBM1* gene. In all of them, except one, reloading of the same PCR product on a newly prepared gel was associated by disappearance of such band. There is no clear explanation for this observation, but errors in sample loading (over loading), uneven gel running, improper gel polymerisation, and improperly flushed wells or combination of these may play a role in producing false results. Differentiation among polymorphic molecules by SSCP is not entirely predictable and the method can result in false negatives, ambiguous results and experimental artifacts (Sheffield et al, 1993).

In one case, the abnormal band shift pattern persisted upon re-running of the same PCR product while it disappeared upon electrophoresis of a newly amplified product from the same exon from the same patient. To know the cause of this condition, the PCR product which showed abnormal band pattern was recovered from low melting point agarose gel. Then 1 ul aliquot was used in asymmetric PCR amplification reactions to generate single stranded DNA. Sequencing of this product revealed the misincorporation of a single base, most probably due to PCR error. The DNA *Taq* polymerase isolated from *Thermus aquaticus* currently used in most PCR studies lacks 3' to 5' proof-reading activity and has an error rate of approximately 1/10000 bases (Shibata 1992). In the previous case, such a PCR error must be produced early in the PCR amplification to make sufficient PCR products detectable by SSCP analysis and DNA sequencing.

It was not possible to screen cDNA since testis RNA is not easily available, especially from patients, and break through transcription in lymphocytes was undetectable.

No data has been published regarding the detection of *RBM1* and *RBM2* point mutations in idiopathic infertile men although several hundred cases have been studied and were only screened for microdeletion in Yq 11 particularly RBM and DAZ gene families. The only study for mutation analysis of RBM was done by Pressor et al (1996). They scanned exons 2-4 for mutation by SSCP on DNA from 30 normal and 60 infertile men. They found a point mutation that alters the

highly conserved RNPs motif (exon 2) in one infertile patient. The mutation was also found in his father. Whether the point mutation found in the patient and his father is a polymorphism or a mutation of significance remains to be determined.

There are a number of potential reasons for this to find more mutations failure. The gene may be unimportant in the process of spermatogenesis although its conservation on the mammalian Y chromosome, testis-specific and germ line nuclear confined pattern argue against this (Elliot et al, 1996). Alternatively, point mutations may not be present or difficult to detect, or in regions of the gene not yet analysed in these patients. RBM is a multicopy family of genes and mosaicism is not easy to demonstrate. In this study it was not possible to find *de novo* point mutation that proves that the *RBM* gene family is involved in the failure of spermatogenesis in all investigated patients. However, although this approach has not provided proof of identify between *RBM* and AZF, it has given basic information.

To provide proof that defects in a gene result in the disease for which it is a candidate remains problematic. The RBM genes provide an example of the difficulty of this approach, which is unfortunately the only one available at present. Firstly, the gene is normally expressed only in the testis, a tissue not readily sampled either the normal population or in patients. This means that the analysis of transcripts *in vivo* is impracticable. Secondly, the gene is not ectopically expressed at a detectable level. Thirdly, the gene has 12 exons, any of which could potentially be mutated in any patient. Fourthly, and most significantly, the gene or sequences related to it are multicopy and give rise to transcripts that vary in the number of copies of the SRGY repeat as well as in a multitude of bases differences. A fifth complication is that some of these changes represent polymorphisms.

Several RBM sequences remain uncharacterised as yet. Until the detailed genomic layout of the gene family members is established, the high degree of similarity between family members and their dispersal throughout the distal part of intervals 5 and 6 are likely to hinder detection of small deletions, and hence, the identification of the individual genes responsible for effects on spermatogenesis.

4.3 Conclusions and recommendations

In conclusion, the most appropriate screening strategies for the detection of molecular pathology are influenced by the expected nature of the mutations, size and structure of the gene in question and the availability of sample (e.g. DNA, fixed chromosomes or RNA). Table 4.2 summarises the sequence of events for mutation detection. Therefore, for each of the four groups studied, a different mutation strategy was chosen and optimised. The present study showed the importance of cytogenetic and FISH approaches in the identification of marker chromosomes, the precise analysis of X and Y chromosomal aberrations as well as the detection of low grade mosaicism. Each of these methods has its own advantages and drawbacks, which should be carefully considered before application.

Classical cytogenetic methods provide more general information on karyotype structure and chromosomal rearrangements but are hardly applicable for identification of marker chromosomes or detection of the exact breakpoint. The latter might be much more easily attained with FISH, which is more rapid, precise, and efficient. Unlike techniques requiring DNA for analysis, in situ hybridisation does not require additional samples, as unstained slides or fixed cell pellets are usually available and amenable to analysis. Additionally, modification of standard FISH techniques allows the use of previously banded slides for immediate determining of the nature of the marker chromosome. This can be crucial for counselling in cases with prenatally detected markers. Most importantly, reliable, high quality DNA probes are easily available for hybridisation, allowing unequivocal determination of the origin and structure of the marker chromosomes.

On the other hand, molecular approaches such as single strand conformational analysis (SSCP), heteroduplex analysis (HA), Chemical cleavage mismatch (CCM) are the most common and rapid methods for detecting minor sequence alterations. DNA sequencing is the most accurate method for screening small genes such as SRY. Multiplex PCR and Southern blot analyses are more applicable for genes or loci prone to deletions such as the AZF locus. These have proven to be a very useful approach to many diseases and are highly recommended methods.

The importance of the combined application of molecular cytogenetic and molecular genetic techniques can enable adequate chromosomal analysis in patients with marker chromosomes or with complex chromosome rearrangements involving the X and Y chromosomes, which can not be confirmed by standard cytogenetic methods.

The strategies of using DNA as template for PCR such as SSCP, HA and DNA sequencing analyses were the main screening methods applied in the present study, and have proved to be a useful method in screening for mutations within the *AR*, *SRY*, and *RBM1* (AZF region) genes.

Molecular investigations indicate that mutations involving the *SRY* gene on the Y chromosome are an infrequent cause of 46,XY gonadal dysgenesis. These mutations have been identified in only about 10-15% of XY females. Accordingly the mutations in the promoter regions might be essential for gene expression. The existence of other sequences crucial for the regulation of the *SRY* gene lying outside the regions studied or other sex reversing gene(s) able to produce indistinguishable phenotypes may be an alternative explanation.

Molecular and FISH techniques are very useful tools in detecting and locating Y sequences in cytogenetically XX males. Testing new Y chromosome markers in XX males will make it possible to identify and narrow the breakpoints in each case which could help in establishing genotype phenotype correlation.

PCR amplification of Y sequences could be effectively used in cases where cytogenetic analysis fails to detect the Y chromosome. Furthermore, it is more specific, and can be used for the precise detection of sequences responsible for the development of testicular tissue (*SRY*), or for regions implicated in the development of gonadoblastoma (e.g., *AMGLY*). This approach considerably improves the diagnosis if compared with the classical cytogenetic analysis and is recommended as an important tool in cases of cytogenetically undetectable markers or in the visualization of cases of unknown origin.

The role of analyses of Y-chromosome microdeletions in evaluating infertile men remains to be determined. Considering the case of PCR-based STS mapping, this screening could become readily available in clinical cytogenetic laboratories. With the advent of intra cytoplasmic sperm injection (ICSI) in the field of reproductive technology, the potential for passing on these defects to

offspring is real and should be considered when infertile couples are counseled. This raises issues of informed consent and ethical concerns. In addition, there is a pressing need for developing mechanisms for worldwide monitoring of ICSI babies for genetic disorders, including Y deletions.

General screening for point mutations in the *RBM* gene family seems to be unwarranted at this time. No data has been published regarding the detection of *RBM* gene family point mutations in idiopathic infertile men. Screening for mutation in gene(s) involved in male infertility alone such as the *DAZ* gene should be considered.

Table 4.2 (a): Methods for mutation detection (Scanning methods)

| Method | Advantages | Limitations |
|---|---|--|
| Cytogenetics | <p>Detects major alteration (>3-5 Mb)</p> <p>General method for chromosome study.</p> <p>Gives a comprehensive view of the 23 pairs of chromosome.</p> <p>Very simple</p> | <p>Can not detect minor alteration.</p> <p>Hardly applicable for marker identification or detection of the exact breakpoints.</p> <p>Requires highly skilled cytogeneticists</p> |
| FISH | <p>Detects major DNA alteration (1-3 Mb)</p> <p>Used as an adjunct to routine cytogenetic to achieve a higher sensitivity and better resolution of chromosome aberrations</p> <p>Simple</p> | <p>Expensive</p> <p>Can not detect minor alteration</p> |
| Southern blot hybridisation | <p>Large scale alteration (>500bp)</p> <p>Very sensitive</p> | <p>Laborious</p> <p>Biohazard (radioactive)</p> |
| Heteroduplex analysis (HA) | <p>Detects minor DNA alteration (1bp)</p> <p>Very simple</p> | <p>Suitable for short DNA sequences (optimal<300 bp)</p> <p>Limited sensitivity</p> <p>Does not show position of the change in the PCR product</p> |
| Single strand conformation polymorphism (SSCP) analysis | <p>Detects minor DNA alteration (1bp)</p> <p>Simple</p> | <p>Suitable for short DNA sequences (optimal<200 bp)</p> <p>Limited sensitivity</p> <p>Does not show position of the change in the PCR product</p> |
| DNA Sequencing | <p>Detects all changes</p> <p>Mutations fully characterised</p> | <p>Laborious</p> |
| <p>Mismatch cleavage</p> <p>(i) Chemical</p> <p>(ii) Enzymatic</p> | <p>Detects minor DNA alteration (1bp)</p> <p>High sensitivity</p> <p>Can easily scan up to 1.7Kb of DNA</p> <p>DNA or RNA may be used as templates</p> <p>Shows position of change</p> | <p>Toxic chemicals</p> <p>Experimentally difficult</p> |

**Denaturing
gradient gel
electrophoresis
(DGGE)**

Detects minor DNA alteration (1bp)
High sensitivity

Choice of primers is critical
GC-clamped primers
Expensive
Does not show position of
the change

**Reverse
transcriptase PCR
(RT/PCR)**

Requires mRNA as a template to check for
splicing errors (deletions and insertions) by
simple gel electrophoresis.
Long segment of peptide coding region can
be analysed.

Detects changes in the
coding sequences only
Gene may not be expressed
in accessible specimens
(tissue specific).
Mutations in promoter and
intronic splice junctions are
not detected.

Because the coding sequence
is a fraction of the size of the
gene, SSCP, HA etc, can be
used as initial screen,
followed by DNA
sequencing

Table 4.2 (b): Methods of testing for a specific mutation

| Method | Application |
|--|--|
| Restriction enzyme digestion of PCR-amplified DNA; check size of products on gel | Only when the mutation creates or abolishes a natural restriction site or one engineered by use of special PCR primers |
| Molecular hybridisation of PCR-amplified DNA to allele-specific oligonucleotides (ASO) on a dot-blot, slot-blot or Southern blot | General method for point mutations |
| PCR using allele-specific primers (ARMS test) | General method for point mutations |
| Check size of expanded trinucleotide repeat | Trinucleotide repeats diseases only; large expansions may require Southern blots, smaller ones can be visualized by PCR-based methods. |
| Multiplex PCR | Applied to loci of known sequences prone to deletions or duplications |

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Appendix I

Table I.1: PCR primers used in analysis of the *AR* gene.

| Exon | Primer sequencer (5' to 3' end) | Size (bp) | Temp | Reference |
|--------|---------------------------------|-----------|-------|------------------------|
| 1A 'F' | GCCTGTTGAACTCTTCTGAGC | 427 | 60 °C | De Bellis et al (1992) |
| 1A 'R' | GCTGTGAAGGTTGCTGTTCCCTC | | | |
| 1B 'F' | CACAGGCTACCTGGTCCTGG | 415 | 60 °C | De Bellis et al (1992) |
| 1B 'R' | CTGCCTTACACAACTCCTTGGC | | | |
| 1C 'F' | GCTCCCACTTCCTCCAAGGAC | 527 | 60 °C | De Bellis et al (1992) |
| 1C 'R' | CGGGTTCTCCAGCTTGATGCG | | | |
| 1D 'F' | CCAGAACACAGAGTGACTCTGCC | 605 | 60 °C | De Bellis et al (1992) |
| 1D 'R' | CCAGAACACAGAGTGACTCTGCC | | | |
| 2 'F' | GCCTGCAGGTTAATGCTGAAGACC | 379 | 60 °C | De Bellis et al (1992) |
| 2 'R' | CCTAAGTTATTTGATAGGGCCTTGCC | | | |
| 3 'R' | TTATCAGGTCTATCAACTCTTGT | 313 | 60 °C | De Bellis et al (1992) |
| 3 'R' | CTGATGGCCACGTTGCCTATGAA | | | |
| 4 'F' | GATAAATTCAAGTCTCTCTTCCT | 360 | 58 °C | De Bellis et al (1992) |
| 4 'R' | GATCCCCCTTATCTCATGCTCCC | | | |
| 5 'F' | TCTTCTTCTCCAGGCTTCCGC | 171 | 58 °C | Lobaccaro et al (1993) |
| 5 'R' | CAGGAGCACTTACTCATTGAA | | | |
| 6 'F' | CTCTGGGCTTATTGGTAAACTTCC | 292 | 58 °C | De Bellis et al (1992) |
| 6 'R' | GTCCAGGAGCTGCCTTTTCCCTA | | | |
| 7 'F' | CTTTCAGATCGGATCCAGCTATCC | 416 | 58 °C | De Bellis et al (1992) |
| 7 'R' | CTCTATCAGGCTGTTCTCCCTGAT | | | |
| 8 'F' | GAGGCCACCTCCTTGTCAACCCTG | 347 | 58 °C | De Bellis et al (1992) |
| 8 'R' | GGAACATGTTTCATGACAGACTGTACACTA | | | |

F stands for forward and 'R' stands for reverse primers.

Table I.2: PCR primers used in analysis of the *SRY* gene.

| Name | Primer sequence (5' to 3' end) | Size | Reference |
|-----------|----------------------------------|--------|----------------------|
| XES7 'F' | CCCGAATTCGACAATGCAATCATATGCTTCTG | 609 bp | Hawkins et al (1992) |
| XES2 'R' | CTGTAGCGGTCCCGTTGCTGCGGTG | | |
| | | | |
| XES10 'F' | GGTGTTGAGGGCGGAGAAATGC | 778 bp | Hawkins et al (1992) |
| XES11 'R' | GTAGCCAATGTTACCCGATTGTC | | |
| | | | |
| SRY A | CCATGAACGCATTCATCGTGTG | 217 bp | Zeng et al (1993) |
| SRY B | GCCTTCCGACGAGGTCGATACT | | |
| | | | |
| SRY C | GAGATCAGCAAGTAGCTGGGA | 226 bp | Zeng et al (1993) |
| SRY D | ACAACCTGTTGTCCAGTTGC | | |
| | | | |
| SRY E | TTTTCAGGACAGCAGTAGAGCA | 251bp | Self designed |
| SRY F | TTGAAAGGTGCCAGAGTTCGA | | |
| | | | |
| SRY G | TGGACAAAGCTGTAGGACAATC | 248 bp | Self designed |
| SRY H | ATAGGCAGGCTCACTTCTGGA | | |

F' stands for forwards and 'R' stands for reverse primers
All sets of primers are annealed at 58 °C.

Table I.3 : PCR Primers used in XX male study.

| Name | Region | Primer sequences | Size* | Temp | Reference |
|-------------|---------------------|--------------------------------|-------|-------|----------------------|
| XES7 'F' | SRY ^a | CCCGAATTCGACAATGCAATCATATGCTTC | 609 | 60 °C | Hawkins et al (1992) |
| XES2 'R' | | CTGTAGCGGTCCCGTTGCTGCGGTG | | | |
| ZFY 5.1 'F' | ZFY | CGAATTCATACCGGCGAGAAGCCATACC | 735 | 61 °C | Barbosa et al (1995) |
| ZFY 6.1 'R' | | AAAGCTTGTAGACACATCGTTAGGG | | | |
| PAR 'F' | PAR-Y | CTGAGAGTGGAAGTGTCGCAG | 1100 | 55 °C | Barbosa et al (1995) |
| PAR 'R' | | GTACTACCTTTAGAAAAGTAGTATTTTCCC | | | |
| Y1 'F' | Y-cent ^b | ATGATAGAACGGAAATATG | 170 | 54 °C | Witt et al (1989) |
| Y2 'R' | | AGTAGAATGCAAAGGGCTCC | | | |

F' stands for forwards and 'R' stands for reverse primers.

* Size in base pairs (bp).

^a Amplifies the HMG box of the *SRY* gene

^b Y- centromeric sequences (DYZ2).

Table I-4: Sets of primers used in the multiplex PCR**.

1A: Y-specific primer pairs of pY6H sequence family

| Name | Locus | Forward | Reverse | Product size(bp) |
|----------|--------|--------------------------|------------------------|------------------|
| Y6D14pr | DYS205 | GGCTAGGTGCCAGCAAGTAGATCA | GTTCTCTTCCCCTGCATCAAG | 134 |
| Y6BaH34p | DYS206 | GCCCTTTGGTAAAAGCG | CTGAATTTGCAAGGGCTGC | 910 |
| H6YP35pr | DYS274 | GGTACACACTCCATCCTGGAC | CTACAGGCTACCTTTTAGGTGG | 226 |
| Y6HP52pr | DYS239 | GGAAGTGGCAGGATTAGCCTTC | GCTCAGAATCTGCGATCAG | 258 |
| Y6PHc54p | ND | GCAGAAGAGTTCAGC | GTGGAGTGCTGCATTAAAGG | 166 |

1B: Primers pairs from DNA loci in Yq11 according to Foote et al (1992) and Scherer et al (1991).

| Name | Locus* | Forward | Reverse | Product size (bp) |
|-----------|---------|---------------------------|---------------------------|-------------------|
| Fr15-11pr | ND | TACCTTGGTTTTGCACCAGACGC | CACCCTCTGTATATGACCTGGC | 313 |
| sY14 | SRY | GAATATTCCCGCTCTCCGGA | GCTGGTGCTCCATTCTTGAG | 472 |
| sY81 | DYS271 | AGGCACTGGTCAGAATGAAG | AATGGAAAATACAGCTCCCC | 209 |
| Sy82 | DYS272 | ATCCTGCCCTTCTGAATCTC | CAGTGTCCACTGATGGATGA | 264 |
| sY84 | DYS273 | AGAAGGGTCTGAAAGCAGGT | GCCTACTACCTGGAGGCTTC | 326 |
| sY86 | DYS148 | GTGACACACACAGACTATGCTTC | ACACACAGAGGGACAACCCT | 320 |
| sY88 | DYS276 | TTGTAATCCAAATACATGGGC | CACCCAGCCATTGTGTTTAC | 123 |
| sY182 | KAL-Y | TCAGAAGTGAAACCCTGTATG | GCATGTGACTCAAAGTATAAGC | 152 |
| sY152 | KAL-Y | AAATCTGTAGTCTCATATCAATCTG | TTACTTGATTTAGCAATAAAAAGG | 183 |
| sY49 | DYS279 | TCATGACAGCCAGGGTATTT | TTTGGACATAGTTTTTGGTCC | 150 |
| sY95 | DYS280 | TCCTACAGATGTCCAAAGTGC | GATGAGTGACCCCAAGAATTG | 303 |
| sY97 | DYS281 | AACTTCATCAGTGTTACATCAAGG | TGTGGCATTGTTGTTATGTGG | 104 |
| sY102 | DYS198 | CACTACCACATTTCTGGTTGG | CGCTGAGTCCATTCTTTGAG | 218 |
| sY105 | DYS201 | AAGGGCTTCTTCTCTTCCTT | AGGGAGCTTAAACTCACCGT | 301 |
| sY109 | DYF43S1 | AGGAGATGTCAGGACTATCAGC | TCCATCCAGCTGGTCATATT | 233 |
| sY117 | DS209 | GTTGGTTCCATGCTCCATAC | CAGGGAGAGAGCCTTTTACC | 262 |
| sY127 | DYS218 | GGCTCACAAACGAAAAGAAA | CTGCAGGCAGTAATAAGGGA | 274 |
| sY134 | DYS224 | GTCTGCCTCACCATAAAACG | ACCACTGCCAAAACCTTCAA | 301 |
| sY143 | DYS231 | GCAGGATGAGAAGCAGGTAG | CCGTGTGCTGGAGACTAATC | 311 |
| sY147 | DYS232 | TTTCTCGTTTGATGATCCTAG | TTAATATGAGAATGAGAACAGATGT | 100 |
| sY149 | DYS1 | TGTCACACTGCCCTAATCCT | TGGTCATGACAAAAGACGAA | 132 |
| sY153 | DYS237 | GCATCCTCATTTTATGTCCA | CAACCCAAAAGCACTGAGTA | 139 |
| sY157 | DYS240 | CTTAGGAAAAAGTGAAGCCG | CCTGCTGTCAGCAAGATACA | 285 |

*Y-DNA locus as defined in Genome Data Base (GDB), Baltimore, MD, USA.

ND = not yet defined in GDB.

** Henegariu et al (1994).

All stes of primers are annealed at 58°C.

Table I-5 : Y-chromosomal STSs used in male infertility study.**

| STS | Forward | Reverse | Product size (bp) |
|---------|---------------------------|--------------------------|-------------------|
| sY231 | ATTGATGTGTTGCCCAAAT | AGAGTGAAC TTAAATCCCAGCC | 149 |
| sY232 | GACTCTACCACTTGGGCTCAATT | AGATGTACCCAAGGCCACTG | 91 |
| sY233 | AGTTAGTAAGCCCCAGTTATCCTCC | TTTGGAAGGACACCTTATTAGCCA | 115 |
| sY236 | CCCCATCGGTAAACCAAATCA | CCCATTGAAGTTTCAAGGTGTCA | 94 |
| sY239a | CATTCATCTTCCCTTTTGAAGG | ATGCAAGTCGCAGGAAATCT | 200 |
| sY240a | TCAAATAGCAGCAATTTAATAT | GCACCTGAAGAGCTGCTTG | 247 |
| sY242 | ACACAGTAGCAGCGGGAGTT | TCTGCCACTAACTGTAAGCTCC | 233 |
| sY243 | GTTTCTTCATAAGCAACCAAATTG | CAGATTATGCCACTGCCCTT | 118 |
| sY245 | TTACTTCCTTAAGTCAAAGCGG | CTGAGACAGCAAGACCAATCC | 101 |
| sY247a | CTGGACAAAGCCTTGGA AAA | CTGCATGTCAATTGTGGGAC | 114 |
| sY248a | CATTGGCATGAATGTGTATTC | CTCTGGGACAAGTGTTCTT | 94 |
| sY249 | GACAAAGGGCTGATGATTTA | CATCACCTTTACTTTTTAAATGG | 114 |
| sY254 b | GGGTGTTACCAGAAGGCAAA | GAACCGTATCTACCAAAGCAGC | 350 |
| sY255 b | GTTACAGGATTCGGCGTGAT | CTCGTCATGTGCAGCCAC | 126 |
| sY257 | AGGTTGTTTGGCCTTGAGC | TCTATGATCTGTACCCGGTGC | 123 |
| sY262 | AGCCACTGCAAGCAACAGA | CCACCATCCCCCTTCTTC | 100 |
| sY267 | GAATGTGTATTCAAGGACTTCTCG | TACTTCCTTCGGGGCCTCT | 102 |
| sY273 | GGTCTTTAAAAGGTGAGTCAAATT | AGACAGAGGGAAC TTCAAGACC | 95 |

a Anneal at 62 °C;otherwise, anneal at 58 °C. b Within *DAZ* gene.

**From Reijo et al (1995).

Table I.6: Primers sequences of 4 different genes involved in male germ cell development that were used in male infertility study **.

| Name | Forward | Reverse | Size (bp) | Temp* |
|-------|----------------------------|----------------------------|-----------|-------|
| RBM1 | ATGCACTTCAGAGATACGG | CCTCTCTCCACAAAACCAACA | 800 | 58 °C |
| RBM2 | AGAGATGCACTTCAGAGG | CCTCTCTCCACAAAACCAACA | 800 | 58 °C |
| DAZ | GGAAGCTGCTTTGGTAGATAC | TAGGTTTCAGTGTTTGGATTCCG | 1300 | 58 °C |
| SPGY1 | TTTCACATACAGCCATTAAGTTTAGC | ACAATTTTGATAGTCTGAACACAAGC | 460 | 58 °C |

Table II.6: Primers designed and used for PCR amplification of RBM (YRRM) gene.

| Exon | Forward | Reverse | Size (bp) | Temp* |
|------|------------------------|------------------------|-----------|-------|
| 2A | CGGCACGACAATGGTAGAA | GGGACCATGTTTCCCAAAT | 113 | 54 °C |
| 2B | AATGGTAGAAGCAGATTGTCA | GATAGGGGACCATGTTTTG | 113 | 56 °C |
| 2C | CGCACGACATGGTAGAG | GGGACCATATTTCCCAAAC | 113 | 56 °C |
| 2D | GTTTACAGCACAATCATCC | TATGGGACCGTGTTTCAC | 100 | 54 °C |
| 4 | GTCTTTGCATGGAAAAGCAATA | TAAACATTACCCAGGTGCC | 190 | 54 °C |
| 5 | GAAATACAGATGATGGTGGA | ATCAGGCAGCATTTACCTTG | 178 | 54 °C |
| 6 | TGAATGTAAAGGTCCCATGT | GGTTTACCCATCGTTAGTTGC | 119 | 54 °C |
| 7 | GCACACAGAAATCATCCAAGT | CAGTACCTATATCCTCTAGAGG | 125 | 60 °C |
| 8 | TATGCATACAGAAATCATCG | AAAAGTTACAGTACCTGTATCC | 136 | 58 °C |
| 9 | CTTCTGCATACAGAAATCGT | CAGAAAAATTACAGTACCTATA | | 58 °C |
| 10 | ACTCGTGCACGCAGAAATCA | ACCTATATCCTCTAGAGGAATG | 127 | 54 °C |
| 11 | CAATTTAGTTATCATGATGGCT | ACCCTTACCGTATCTCTGAA | 105 | 58 °C |
| 12 | AACCCTGCAGGGACCTCTCA | CTACCTCTCTCCACAAAACCAA | 437 | 58 °C |

* Annealing temperature.

**From Vogt et al (1996).